PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{5} : C12N 9/00, A61K 31/70 C07H 21/00, C12P 19/34	A1	(11) International Publication Number: **WO 93/15187** (43) International Publication Date: 5 August 1993 (05.08.93)

(72) Inventors: USMAN, Nassim ; 3 Lawrence Street, Boston, MA 02116 (US). CEDERGREN, Robert, J. ; 4675 Christophe Colomb, Montreal, Quebec H2J 3G7 (CA). PERREAULT, Jean-Pierre ; 3291 Hanna Street, Rawden, Quebec J0K 1S0 (CA). YANG, Jing-Hua ; 2676 Monpetit Avenue, Montreal, Quebec H3T 1J6 (CA). RICH, Alexander ; 2 Walnut Avenue, Cambridge, MA 02116 (US).

(54) Title: NUCLEOZYMES

(57) Abstract

Nucleozymes containing ribonucleotides and deoxyribonucleotides or nucleic acid analogues are described herein. The nucleozymes have catalytic activity and are significantly more resistant to degradation than their all-RNA ribozyme counterparts. Also described are methods for preparing the nucleozymes along with methods of using nucleozymes, e.g., as therapeutic agents.

CORRECTED
VERSION *

PCT

under D Number (72) "Inventors; and" and
(75) "Inventors/Applicants (for US only)", add
"HEIDENREICH, Olaf |DE/DE|; Karolinenweg
15, D-3400 Göttingen (DE)"

86139/91

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 9/00, 15/00, A61K 31/00	A1	(11) International Publication Number: WO 92/07065 (43) International Publication Date: 30 April 1992 (30.04.92)

(21) International Application Number: PCT/EP91/01811

(22) International Filing Date: 23 September 1991 (23.09.91)

(30) Priority data:
PCT/EP90/01731 12 October 1990 (12.10.90) EP
(34) Countries for which the regional
or international application
was filed: AT et al.

(71) Applicant (for all designated States except US): MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Bunsenstraße 10, D-3400 Göttingen (DE).

(72) Inventors; and
(75) Inventors/Applicants (for US only) : ECKSTEIN, Fritz [DE/DE]; Holundersteg 6, D-3400 Göttingen (DE). PIEKEN, Wolfgang, A. [DE/DE]; Auf der Wessel 22, D-3400 Göttingen (DE). BENSELER, Fritz [DE/DE]; Heinrich-Warnecke-Straße 4, D-3400 Göttingen (DE). OLSEN, David, B. [US/US]; Merck, Sherp & Dohme, Research Laboratory, WP 42-300, Westpoint, PA 19486 (US). WILLIAMS, David, M. [GB/DE]; Wiesenstraße 15, D-3400 Göttingen (DE). HEIDENREICH, Olaf |DE/DE|; Karolinenweg 15, D-3400 Göttingen (DE).

(74) Agents: WEICKMANN, H. et al.; Postfach 86 08 20, Möhlstrasse 22, D-8000 München 80 (DE).

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU+, TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

640074

(54) Title: MODIFIED RIBOZYMES

(57) Abstract

The present invention refers to an RNA molecule with catalytic activity comprising at least one modified nucleoside, wherein the hydroxy group at the 2'-position of the ribose sugar is replaced by a modifier group, selected from halo, sulfhydryl, azido, amino, monosubstituted amino and disubstituted amino groups, a process for the preparation of modified RNA molecules and the use of modified RNA molecules as therapeutic agents and biocatalysts.

Modified Ribozymes

SPECIFICATION

Certain naturally occuring ribonucleic acids (RNAs) are
subject to self-cleavage. The first reported example is the
cleavage of the ribosomal RNA precursor of the protozoan
Tetrahymena (for a review see Cech, Ann.Rev.Biochem. 59
(1990), 543-568) which requires guanosine as cofactor. A
number of examples of RNA cleavage have been subsequently
discovered in viroid, virusoid and satellite RNAs (for
reviews see Sheldon et al. in Nucleic Acids and Molecular
Biology (1990) Vol. 4, pg. 227-242, ed. F. Eckstein and
D.M.J. Lilley, Springer Verlag Berlin Heidelberg; Symons,
TIBS 14 (1989), 445-450). These cleavages involve site-
specific breakage of a phosphodiester bond in the presence of
a divalent cation such as Mg^{2+}, generating a 5'-hydroxyl and
a 2',3',-cyclic phosphodiester terminus. Sequence analysis
around the site of self-cleavage of several of such RNAs has
led to the identification of a common structural feature
essential for cleavage which was named a "hammerhead"
structure (Hutchins et al., Nucleic Acids Res. 14 (1986)
3627-3640). This structure consists of three helices and 13
conserved nucleotides (framed in below scheme) which form a
three dimensional structure amenable to cleavage at one
particular position. The self-catalyzed cleavage is normally
an intramolecular process, i.e. a single RNA molecule
contains all the functions necessary for cleavage. However,
Uhlenbeck (Nature 328 (1987), 596-600) has demonstrated that
this hammerhead structure does not have to be embodied in one
strand but can be made up of two strands. These two strands
combine to form the hammerhead structure which leads to
phosphodiester bond cleavage (indicated by an arrow) in one
of the strands (strand S) whereas the other (strand E)
remains unaltered and can participate in many cleavage
reactions. This strand meets the definitions of an enzyme and
is called a ribozyme. Whereas the framed sequences (below
scheme) are conserved the others may vary provided that the

3

viral genes and particular ndogenous genes. This requires
the construction of a RNA molecule which is able to form a
hammerhead or a hairpin structure with the target RNA and to
cleave this at a predetermined position. A first application
to the inhibition of the HIV-1 virus by this strategy has
been reported (Sarver et al., Science 247 (1990), 1222-1224).
Other examples of the action of targeted hammerhead ribozymes
in vivo are Cammeron and Jennings (Proc.Natl.Acad.Sci. USA 86
(1986), 9139-9143) and in vitro Cotten et al. (Mol.Cell.Biol.
9 (1989), 4479-4487).

Further, other useful catalytic properties of ribozymes are
known, e.g. dephosphorylase and nucleotidyl transferase
activities (see Patent Application WO88/04300). Therein RNA
enzymes are disclosed which are capable of dephosphorylating
oligonucleotide substrates with high sequence specifity,
which distinguishes them from known protein enzymes. RNA
molecules also can act as RNA polymerases, differing from
protein enzymes in that they use an internal rather than an
external template. Thus, various heteropolymers can be
constructed by variant RNA enzyme forms. This enables the
formation for example of messenger RNA molecules for
particular proteins or peptides. Furthermore, Herschlag and
Cech (Nature 344, (1990), 405-409) describe an RNA enzyme
with DNase acitivity.

To be useful as a therapeutic agent the RNA enzyme has to be
introduced into target cells. There are a priori two methods
for delivery of the ribozyme into the target cells:
(a) exogenous delivery of a preformed synthetic RNA;
(b) endogenous transcription of a ribozyme-coding gene
    located on a plasmid.

A great disadvantage of method (a) resides in the very low
stability of RNA molecules under physiological conditions due
to their fast degradation by a variety of ribonuclease

5

activity comprises a sequence specific endoribonuclease
activity. More preferably the RNA is a hammerhead ribozyme as
described above. Especially preferred is that the ribozyme
can combine with another RNA strand to form a hammerhead
structure consisting of two strands, wherein the modified RNA
strand is the E strand as described above.

Although a hammerhead ribozyme is especially preferred, other
RNA enzymes are encompassed also by the present invention,
e.g. the Tetrahymena ribozyme (Cech, Ann.Rev.Biochem. 59
(1990), 543-568) in naturally occuring form or a shortened
form thereof (Zang et al., Biochemistry 27 (1988), 8924-
8931), and especially the Hairpin RNA (Hampel et al., Nucleic
Acids Res. 18 (1990) 299-304) or RNA containing proteins such
as the RNase P (Baer et al., in Nucleic Acids & Molecular
Biology (1988), Vol. 3, pp 231-250, ed. F. Eckstein and
D.M.J. Lilley, Springer Verlag Heidelberg), the telomerase
(Greider and Blackburn, Nature 337 (1989), 331-337).

The incorporation of a modifier group at the 2'-position of
the ribose sugar appears also to be particularly useful for
RNA with new functions either derived at by a procedure that
depends on alternate cycles of selection (Tuerk and Gold,
Science 249 (1990), 505-510; Ellington and Szostak, Nature
346 (1990), 818-822) or any other method .

The modifier group replacing the hydroxy group at the 2'-
position of the ribose sugar is selected from halo,
sulfhydryl, azido, amino, monosubstituted amino, and
disubstituted amino groups. The halo group can be a fluoro,
chloro, bromo or iodo group, wherein the fluoro group is
preferred. The substituents of the substituted amino group
are preferably C_1-C_3 alkyl and or hydroxyalkyl groups. Most
preferably the modifier group is a halo or an unsubstituted
amino group.

7

5-position with halo or C_1-C_5 alkyl groups, especially bromo
or methyl groups. Most preferably the nucleotide base
attached to the modified ribose sugar is uracil.

The modified nucleosides which are incorporated into a RNA
molecule are either previously described compounds or
compounds which can be prepared in analogy to known
compounds. The mostly preferred fluoro and amino analogs of
ribonucleosides have been described previously, 2'-deoxy-2'-
fluorocytidine (Doerr & Fox, J.Org.Chem. 32 (1967), 1462;
Mengel & Guschlbauer, Ang.Chem. 90 (1978), 557-558); 2'-
deoxy-2'-fluoroadenosine (Ikehara & Miki, Chem.Pharm.Bull. 26
(1978), 2449-2453), 2'-deoxy-2'-fluorouridine (Codington et
al., J.Org.Chem. 29 (1964), 558-564), 2'-deoxy-2'-
aminouridine (Verheyden et al., J.Org.Chem. 36 (1971), 250)
and 2'-deoxy-2-aminocytidine (Verheyden et al. (1971) supra).
For the synthesis of some of these compounds more recent
synthetic procedures can be employed. The 2'-deoxy-2'-
fluorocytidine can be prepared from 2'-deoxy-2'-fluorouridine
by the method of Sung (J.Org.Chem. 47 (1982), 3623-3628). The
same method can be used for the transformation of 2'-deoxy-
2'-azidouridine to 2'-deoxy-2'-azidocytidine (Verheyden et
al. (1971), supra). The latter can be reduced to 2'-deoxy-2'-
aminocytidine by the method of Mungall et al. (J.Org.Chem. 40
(1975), 1659).

The synthesis of the 2'-deoxy-2'-fluoronucleoside 5'-
triphosphates can be carried out either according to Ludwig
(Acta Biochim. et Biophys. Acad.Sci.Hung. 16 (1981), 131-133)
or Ludwig and Eckstein (J.Org.Chem. 54 (1989), 631-635). The
2'-deoxy-2'-aminouridine and -cytidine 5'-triphosphates can
be prepared as described for the diphosphates by Hobbs et al.
(Biochemistry 12 (1973), 5138-5145) with the modification
that pyrophosphate is employed instead of phosphate. The 2'-
deoxy-2'-fluoronucleoside 3'-phosphoramidites for automated
oligonucleotide synthesis can be prepared by the method of

9

nucleosides are modified and which are unmodified) which is
d signated as a so-called "selectiv modification pattern".
An RNA comprising selective modification pattern is a
molecule wherein nucleosides at specifically selected
locations can be modified while nucleosides at other
specifically selected locations can be unmodified. For
instance, nucleotides which are known to be hypersensitive
sites for ribonucleases (e.g. due to the secondary structure
of the RNA molecule) should be modified to achieve an
extended life time of the RNA molecule. An example for a
ribonuclease-hypersensitive site is provided at position 21
of ribozyme E1. As shown in Fig. 3 the RNA molecule is
cleaved at this position by RNase A with very high intensity.

Still a further embodiment of the present invention is a RNA
molecule additionally comprising at least one modified
internucleotidic phosphodiester linkage. Examples for
suitable modified phosphodiester linkages are methyl
phosphonate groups or phosphorothioate groups, the latter
being especially preferred. Preferably at least the 5'-
terminal phosphodiester linkage and/or the 3'-terminal
phosphodiester linkage of the RNA molecule is modified. More
preferably the 5'-terminal phosphodiester linkage and the
last three 3'-terminal phosphodiester linkages are modified.

It was found, that the presence of modified internucleotidic
linkages alone was not sufficient to provide increased
stability against degradation. However, the combined presence
of 2'-modified ribose sugars together with modified
internucleotidic linkages showed an additive stability
enhancing effect. A more than fiftyfold increase in stability
confered by both modifications outweighs the decreased
efficiency in cleavage compared to a unmodified ribozyme.

precursors. After the coupling of the precursor to the RNA
chain has taken place the subsequent oxidation step, however,
is not performed with iodine, as in the case of non-modified
linkages, but with sulfur or a sulfurating agent, whereby the
phosphorothioate group is obtained.

The chemical synthesis of modified RNA molecules is carried
out in analogy to that of unmodified RNA or DNA molecules,
which is known in the art. More specifically the RNA
synthesis is carried out by chemical synthesis on solid
support involving the stepwise addition of the respective
nucleotide precursors. After having synthesized an RNA
product of the desired length, the RNA is removed from the
solid support by conventional means and purified, preferably
by gel electrophoresis. Alternatively the chemical RNA
synthesis can also be carried out by any other known
technique without using a solid support. E.g. the RNA can be
synthesized in a soluble form and subsequently purified by
means known in the art.

When the 2'-amino modifier group is incorporated into the RNA
chain it has to be protected before the phosphitylation
reaction (i.e. the preparation of the nucleotide precursor)
and for subsequent use in the coupling reactions. For this
purpose the trifluoroacetyl group is preferably used as a
protecting group, because it is stable during the cycles of
synthesis on the nucleic acid synthesizer and is removable
under the conventional treatment with ammonia.

Alternatively the synthesis of the RNA chain can be carried
out by transcription from a nucleic acid template by an
appropriate nucleic acid polymerase. Preferably the template
is a DNA template and the nucleic acid polymerase is a DNA
dependent RNA polymerase. Mor preferably the DNA dependent
RNA polymerase is selected from the group consisting of T7,
T3 and SP6 polymerases, which are highly processive

rang of Km variation observed for hammerhead systems with
different base composition (Fedor & Uhlenbeck, supra). In
addition, very surprisingly the incorporation of a single 2'-
aminouridine immediately 5' of the site of cleavage in the
substrate increased the kcat markedly (table 1), so that it
is conceivable to produce ribozymes of enhanced activity by
the selective introduction of 2'-modified nucleosides at
specific sites. These results definitely show that there is
no requirement for the presence of 2-hydroxyl groups
throughout the enzyme part of the hammerhead structure for
catalytic activity but that the modifications according to
the present invention are tolerated at least in certain
positions. In contrast, the incorporation of only 15 % 2'-
deoxynucleotides into a hammerhead ribozyme is reported to
decrease the catalytic efficiency by two orders of magnitude,
while not affecting the K_m (Perreault et al. (1990), supra).
Since the rate of cleavage is determined by the angle of
attack of the 2'-hydroxyl on the phosphorus at the site of
cleavage, it is greatly influenced by the overall structure
of the hammerhead system. Thus, the observed influence of 2'-
modifications on the rate supports the notion that the 2'-
fluoro analogs adopt a structure more similar to that of
ribonucleotides than that of deoxyribonucleotides. This
apparently also holds for the amino analogs. The other 2'-
modified nucleosides according to the present invention
exhibit similar catalytic activity.

A still further object of the present invention is the use of
RNA molecules with catalytic activity comprising at least one
modified nucleotide, as therapeutic agents, especially for
the specific cleavage of viral or other foreign genetic
material or transcripts from viral or other foreign genetic
material, or as biocatalyst in biochemical or
biotechnological processes. For these purposes the RNA
molecules of the present invention seem to be more suitable
than their unmodified analogs, because of their increased
stability against chemical and/or enzymatical cleavage.

15

EXAMPLES

<u>Example 1</u>

Preparation of oligoribonucleotides

Automated synthesis of oligoribonucleotides: Automated
oligoribonucleotide synthesis was carried out with an Applied
Biosystems 380B DNA Synthesizer on a 1 μmol scale using the
monomeric ribonucleotide phosphoramidites supplied by
Milligen/Biosearch. Control pore glass columns with the
ribonucleoside coupled to it were either from Milligen/Bio-
search or Peninsula. The oligomers were worked up according
to the specifications of the supplier of the ribonucleotide
phosphoramidites (Milligen/Biosarch). After removal of the
protecting groups the oligoribonucleotides were concentrated
by spin dialysis on Amicon filter membranes centricon 10 and
ethanol precipitated. The dried pellets were taken up in
50 μl water and subjected to PAGE. Bands were visualized by
UV shadowing, cut out and the RNA was isolated by eluting at
37°C overnight in buffer (0.25 M ammonium acetate, 10 mM
TRIS/HCl pH 8.0, 1 mM EDTA) (Fedor & Uhlenbeck, PNAS USA 87
(1990), 1668-1672). Concentrations were determined using the
extinction coefficient per nucleotide of 6600 $M^{-1}cm^{-1}$ given
in the literature (Fedor & Uhlenbeck 1990). Aqueous solutions
of the oligoribonucleotides were stored at -20°C.

Construction of plasmids containing templates for run off
transcription:

The following oligodeoxynucleotides were synthesized for the
plasmid construction by the phosphoramidite method with an
Applied Biosystems 380B DNA synthesizer:

RS2-T,5'-d(GATATCCTGACTCCCTATAGTGAGTCGTATTA)-3; RS2-C,5'-
d(TAATACGACTCACTATAGGGAGTCAGGATATCTGCA)-3'; RE1-T,5'-
d(GGAGTTTCGGCCTAACGGCCTCATCAGAGGACCCTATAGTGAGTCGTATTA)-3' and
RE2-C,5'-
d(TAATACGACTCACTATAGGGTCCTCTGATGAGGCCGTTAGGCCGAAACTCCTGCA)-3'.

Figure 1 shows autoradiographs of T7 RNA polymerase run ff transcriptions of pUCRS after PAGE. A: The transcription was performed on a 150 μL scale in the presence of 20 mM $MgCl_2$ and 2.5 mM each of the four nucleoside triphosphates at 37°C for 3 h. The reaction mixture was dephosphorylated with alkaline phosphatase and 5'-^{32}P-labeled by reaction with polynucleotide kinase and [γ-^{32}P]-ATP. The labeled transcription mixture was subjected to PAGE. B: The transcription was performed on a 150 μL scale at 37°C for 3 h in the presence of 20 mM $MnCl_2$, 0.5 mM ATP, 25 μCi [α-^{32}P]-ATP, 2.5 mM CTP and GTP, and 2.5 mM 2'-fluorouridine triphosphate. The transcription mixture was directly subjected to PAGE. The asterisks mark ^{32}P-labeled phosphates. 'N' denotes any nucleotide added by T7 RNA polymerase beyond the full length of the template DNA (c.f. Milligan and Uhlenbeck, Meth.in Enzymology 180A (1989), 51-62).

Figure 2 shows an autoradiograph of T7 RNA polymerase run off transcripts of pUCRE 16 containing 2'-aminouridine after PAGE. Lane 1: 2'-aminouridine containing 34-mer marker E3, synthesized chemically. Lane 2: The transcription was performed on a 150 μl scale at 37°C for 3 h in the presence of 20 mM $MgCl_2$, 60 μCi [α-^{32}P]ATP, 1 mM CTP and GTP, and 1 mM 2'-aminouridine triphosphate. The transcription mixture was directly applied PAGE.

Preparation of oligoribonucleotides: The following oligoribonucleotides were prepared
a.) by run off transcription (sequences given without the 5'-triphosphate):

E1, 5'-GGGUCCUCUGAUGAGGCCGUUAGGCCGAAACUCC-3';

E2, 5'-GGG(2'-FU)CC(2'-FU)C(2'-FU)GA(2'-FU)GAGGCCG(2'-FU)(2'-FU)AGGCCGAAAC(2'-FU)CC-3' and

E3, 5'-GGG(2'-NH_2U)CC(2'-NH_2U)C(2'-NH_2U)GA(2'-NH_2U)GAGGCCG(2'-NH_2U)(2'-NH_2U)AGGCCGAAAC(2'-NH_2U)CC-3';

Total degradation of oligoribonucleotides by RNAse A: The oligoribonucleotides S1 and S2 were digested with RNase A after $5'-^{32}P$ labeling according to the following protocol: The oligomer (8.5 μM in a final volume of 20 μl) was reacted with 1.25×10^{-3} Units of RNAse A in buffer containing 50 mM TRIS/HCl pH 7.5 and 10 mM $MgCl_2$ for 10 min at 37°C. Products were analyzed by PAGE.

Figure 3 shows an autoradiograph of partial Ribonuclease A cleavage of 5'-labeled run off transcripts E1 and E2 separated by PAGE. Conditions as described before. The numbered lanes correspond to 1) no enzyme added, 2) 2×10^{-4} units RNase A, 3) 3×10^{-5} units RNase A, 4) 8×10^{-6} units RNase A, 5) 16×10^{-7} units RNase A. Base numbering was facilitated by counting the bands of a Mn^{2+} mediated cleavage of the unmodified transcript (10 μmoles RNA heated to 90°C for 3 min in 10 mM $MnCl_2$). The circled numbers indicate the bands expected from RNase-A susceptible cleavage positions. Arrows indicate the bands that arise from cleavage 3' to uridine and which are absent in the lanes where 2'-fluorouridine containing ribozyme was cleaved.

Figure 4 shows an autoradiograph of the total degradation of S1 and S2 by RNase A after PAGE. Details of the reaction are as described above. Lane 1: total digestion of 12-mer S2; lane 2: total digestion of 12-mer S1; lane 3: cleavage ladder of the 34-mer E1 by reaction with 20 mM $MnCl_2$ at 90°C for 3 min as a length standard. The product of cleavage of S2 is 1 nucleotide longer than that of S1 indicating the presence of 2'-aminouridine at position 6.

21

However, potentially more interesting for future applications
is the question whether the presence of this modification in
the enzyme part of the ribozyme will interfere with its
catalytic activity. Thus, the reaction of the 2'-
fluorouridine-containing ribozyme E2 with the unmodified
substrate S1 was investigated. Indeed, the gel analysis
indicated that the substrate was cleaved with similar
efficiency as the pair E1 and S1. The catalytic constants of
the 2'-fluorouridine-containing ribozyme E2 were determined
(Fig. 6) and compared to those of the unmodified ribozyme E1.
This comparison reveals that the second order rate constant
for the former (k_{cat}/K_m = 0.0026 nM^{-1}) is one order of
magnitude smaller than that of the latter (k_{cat}/K_m = 0.023
nM^{-1}) (Fedor & Uhlenbeck (1990), supra) (Table 1). This
decrease in catalytic efficiency is primarily due to a
decrease in the rate of cleavage, whereas the Km values for
both ribozymes is nearly identical. This reduced rate of
cleavage, however, lies well within the range of cleavage
efficiencies observed for various hammerhead systems with
different base compositions (Fedor & Uhlenbeck (1990),
supra). Hammerhead ribozyme reactions can be carried out with
$MgCl_2$ as well as with $MnCl_2$ as metal ion cofactor, where the
half life of cleavage is decreased in the presence of the
latter cofactor by about 10 fold (Uhlenbeck, Nature 328
(1987), 596-609). Such a decrease in the half life of the
substrate under cleavage conditions upon switching from Mg^{2+}
to Mn^{2+} was also observed for the reaction of 2'-
fluorouridine-containing enzyme E2 with substrate S1. Thus
the metal ion requirement for the cleavage reaction is not
altered by the incorporation of 2'-fluoronucleotide analogs.

The effect of the presence of 2'-aminouridine in the ribozyme
was also investigated. When the 2'-aminouridine containing
ribozyme E3 is reacted with nonmodified substrate S1, the
catalytic efficiency drops an order of magnitude to k_{cat}/K_m =
0.0015 nM^{-1}. This decrease in efficiency is clearly due to a

23

range of variations observed for hammerhead systems of
different bas composition. It also becomes evident that it
is possible to increase the catalytic efficiency by
selectively introducing 2'-modifications at specific
positions. While the latter effect was demonstrated for the
substrate oligoribonucleotide, it is anticipated that a
similar influence on catalysis can be found for selective
modifications in the enzyme.

Figure 5 shows an autoradiograph of the cleavage of 2'-
fluorouridine and ^{32}P-AMP-containing substrate S3 by ribozyme
E1. The cleavage reaction was performed in the presence of 10
mM MgCl$_2$ in 50 mM TRIS/HCl, pH 7.5 on a 40 μl scale at 25°C.
The concentration of E1 and S3 was 2.5 μM and 7.5 μM,
respectively. All other details are as described above (c.f.
Determination of Cleavage Kinetics). At the indicated times
10 μl aliquots were transferred into 10 μl water and 10 μl
urea stop mix prior to PAGE. Lane 1: reaction after 0.5 min;
lane 2: reaction after 15 min; lane 3: reaction after 30 min.
The asterisks mark ^{32}P-labeled phosphates.

Figure 6 shows an Eadie-Hofstee plot of the ribozyme reaction
of E2 with S1. The cleavage reaction were performed on a
20 μl scale in the presence of 10 mM MgCl$_2$ with a 10 nM
concentration of E2 and concentrations of S1 of 50 nM,
100 nM, 200 nM, 400 nM, 500 nM, and 700 nM. After 7 min 10 μl
aliquots were transferred into 10 μl water and 10 μl urea
stop mix prior to PAGE. It was established previously that
these time points fall within the linear range of initial
velocities. The autoradiographs were evaluated by integration
of their optical density on a laser densitometer.

Figure 7 shows an Lineweaver-Burk plot of the ribozyme
reaction of E3 with S1. The cleavage reactions were performed
on a 20 μl scale in the presence of 10 mM MgCl$_2$ with a 10 nM

25

catalyzed cleavage. Hammerhead ribozymes targeted against this site were chemically synthesized. The nucleotide sequence of the unmodified hammerhead enzyme RE115 is given in Fig. 9.

Cleavage Kinetics with LTR RNA: k_{cat}/K_m values were determined under single turnover conditions. Ribozymes were preincubated at 75°C for 1 min in the presence of 50 mM Tris-Cl pH 7.5 followed by 5 min of incubation at 37°C. MgCl$_2$ was added to a final concentration of 10 mM and the solutions were again incubated for 5 min at 37°C. LTR RNA was directly used as an aqueous solution. The reaction mixture (10 μl) contained between 20 nM and 1 μM ribozyme, 50 mM Tris-Cl pH 7.5 and 10 mM MgCl$_2$. The reaction was started by addition of LTR RNA to a final concentration of 10 nM. After 1 hour at 37°C the reaction was stopped by addition of 10 μl stop mix and analysed by 4 % PAGE (40 cm long, 8 M urea). After 1 h electrophoresis at 50 W followed by autoradiography the fraction of noncleaved LTR RNA was determined by laser scanning densitometry. k_{cat}/K_m values were obtained by plotting the remaining fraction of LTR RNA (Frac S) against the ribozyme concentration ([RE]) according to the following equation:

$$k = \frac{\ln(\text{FracS})}{t} = [RE]\frac{k_{cat}}{K_m} \, ,$$

where k is the observed reaction rate and t is the reaction time of 1 h.

In order to investigate the influence of chemical modifications on the catalytic efficiency of a ribozyme several analogs of RE115 containing 2'-fluoro or 2'-deoxy substitutions and/or terminal phosphorothioate linkages were synthesized. Whereas 2'-fluorocytidine substitutions hat no

27

Example 5
Stability of oligoribonucleotides

The ribozymes of Example 4 were examined for their stability
against nuclease digestion.

Test conditions:
Molt 4 clone 8 cells (kindly supplied by E. Jurkiewicz,
Deutsches Primatenzentrum, Göttingen) grown in medium RMPI
1640 to a cell density of about 10^6 cells/ml were centrifuged
at 1000 g for 5 min in a Heraeus Minifuge. $5'-^{32}P$-labeled
ribozymes were pre-heated for 1 min at 90°C, chilled on ice,
added to the cell supernatant to a final concentration of
300 nM and incubated at 37°C. Aliquots were taken at the
indicated time points and analysed by 20 % PAGE containing
8 M urea followed by autoradiography.

Results:
More than 80 % of RE115 was degraded after 2 min incubation
in the cell supernatant as indicated by denaturing PAGE. For
RE115(S) similar results were obtained. However, no
degradation of RE115(FC,FU,S) within 1 hour was observed. A
comparison with the rate of degradation of the unmodified
ribozyme indicates that the combination of 2'-modified
pyrimidine nucleosides and terminal phosphorothioate linkages
results in an estimated increase of more than fiftyfold of
ribozyme stability against digestion by nucleases present in
T cell supernatant. 2'-modified ribozymes without
phosphorothiate group show a stability which is about two
times lower than the stability of RE115 (FC,FU,S).

29

8.   RNA according to claim 7, wherein the substituted nucleotide base is selected from the group consisting of xanthine, hypoxanthine, 2,6-diamino purine, 2-hydroxy-6-mercaptopurine and purine bases substituted at the 6-position with sulfur or pyrimidine bases substituted at the 5-position with halo or C_1-C_5 alkyl groups.

9.   RNA according to claim 7, wherein the nucleotide base attached to the modified ribose sugar is a base naturally occuring in RNA.

10.   RNA according to claim 9, wherein the nucleotide base attached to the modified ribose sugar is a pyrimidine base.

11.   RNA according to any of the preceding claims, wherein all nucleotide bases of one specific kind are attached to a modified ribose sugar.

12.   RNA according to claim 11, wherein all uracil nucleotide bases are attached to a modified ribose sugar.

13.   RNA according to claim 11, wherein all cytosine nucleotide bases are attached to a modified ribose sugar.

14.   RNA according to any one of the claims 1 - 10, wherein all nucleotide bases of two specific kinds are attached to a modified ribose sugar.

15.   RNA according to claim 14, wherein all cytosine and uracil nucleotide bases are attached to a modified sugar.

24.   RNA according to any of the claims 20 - 23, wherein the 5'-terminal phosphodiester linkage and the last three 3'-terminal phosphodiester linkages are modified.

25.   Process for the synthesis of an RNA molecule with catalytic activity, comprising:
incorporating into an RNA chain at least one modified nucleotide, wherein the hydroxy group at the 2'-position of the ribose sugar is replaced by a modifier group, selected from halo, sulfhydryl, azido, amino, monosubstituted amino and disubstituted amino groups.

26.   Process according to claim 25, wherein the modifier group is a halo or an amino group.

27.   Process according to claim 25 or 26, wherein the halo group is a fluoro group.

28.   Process according to any of the claims 25-27, wherein the synthesis of the RNA chain is carried out by chemical synthesis from nucleotide precursors on solid support, removing the RNA product from said solid support and purifying the removed RNA product.

29.   Process according to any of the claims 25-27, wherein the synthesis of the RNA chain is carried out by chemical synthesis from nucleotide precursors in solution and purifying the RNA product.

30.   Process according to claim 28 or 29, wherein the respective phosphoramidites or H-phosphonates are used as nucleotide precursors.

39.   Process according to any of the claims 34 - 37, wherein the modifier group is a amino, monosubstituted amino, or disubstituted amino group and the synthesis of the RNA chain is carried out in presence of Mg^{2+} ions.

40.   Use of an RNA according to any of the claims 1 - 24 as a therapeutic agent or a biocatalyst.

41.   Therapeutic agent comprising as active ingredient an RNA according to any of the claims 1 - 24, optionally together with convenient fillers, adjuvants, carriers and diluents.

42.   Process of preparing a therapeutic agent, wherein the therapeutic agent comprises as active ingredient an RNA according to any of the claims 1 - 24, optionally together with convenient fillers, adjuvants, carriers and diluents.

Dated this 2nd day of March, 1994

Max-Planck-Gesellschaft zur Forderung der Wissenschaften e.V.

By its Patent Attorneys
Davies   Collison   Cave

Fig. 2

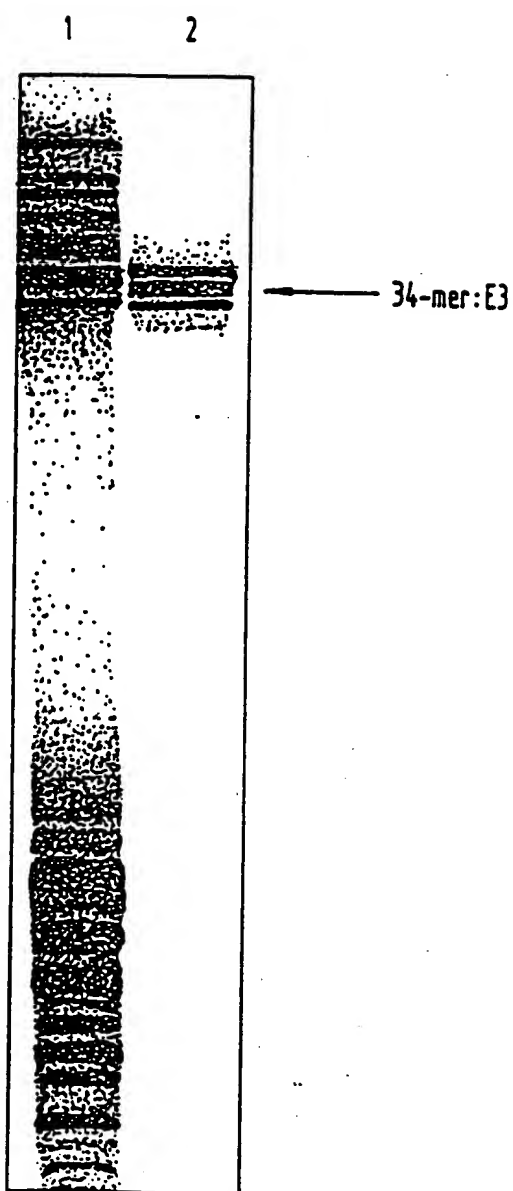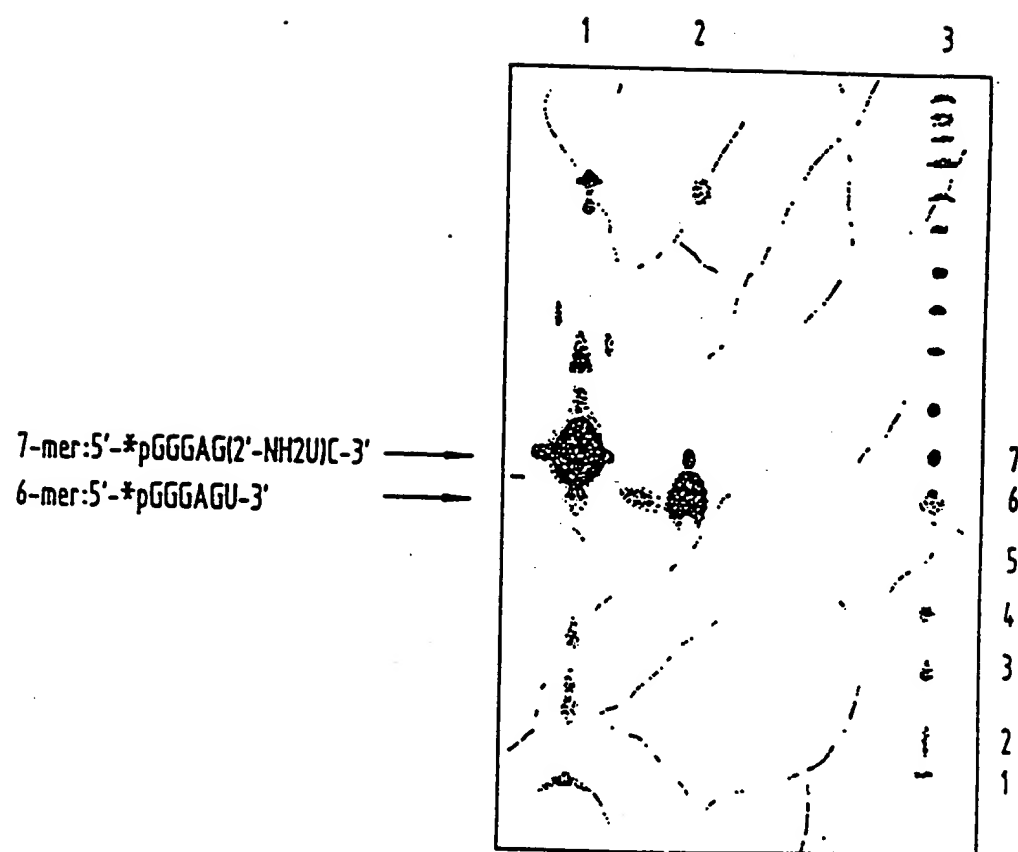# Fig.4

7-mer:5'-*pGGGAG(2'-NH2U)C-3' ⟶

6-mer:5'-*pGGGAGU-3' ⟶

Fig.6

Ribozyme reaction of E2+S1
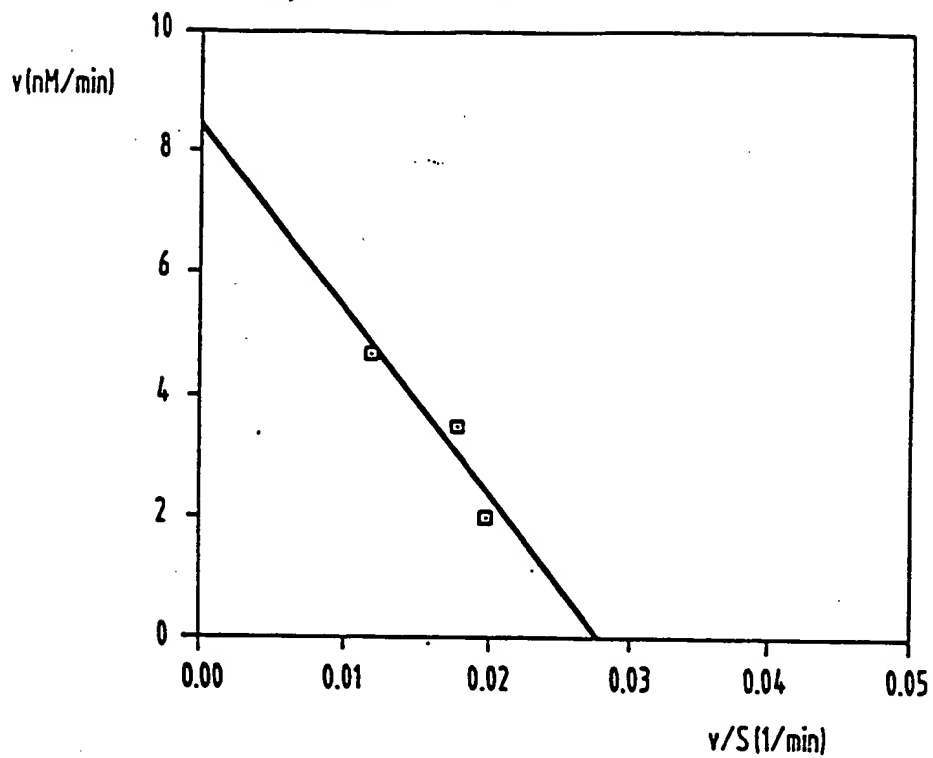
Fig.7
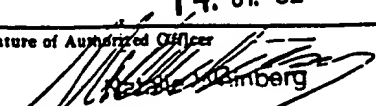
Ribozyme reaction of E3+S1

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER   (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5	C 12 N 9/00	C 12 N 15/00	A 61 K 31/00

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.Cl.5	C 12 N A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category °	Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²	Relevant to Claim No.¹³
A	Trends in Biotechnology, volume 8, no. 7, July 1990, Elsevier Science Publishers Ltd, (Cambridge, GB) J.J. Rossi et al.: "RNA enzymes (ribozymes as antiviral therapeutic agents", pages 179-183, see the whole article	1,26
A	Trends in Biotechnology, volume 8 no. 7, July 1990, Elsevier Science Publishers Ltd (Cambridge, GB) M. Cotten: "The in vivo application of ribozymes", pages 174-178, see the whole article	1,26
A	EP,A,0321201 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 21 June 1989, see the whole document, especially page 5 -/-	1,26

° Special categories of cited documents :¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25-11-1991	14. 01. 92

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Imberg

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 20/12/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0321201	21-06-89	AU-A-	2800789	19-07-89
		WO-A-	8905852	29-06-89
WO-A- 8804300	16-06-88	US-A-	4987071	22-01-91
		EP-A-	0291533	23-11-88
		JP-T-	1501445	25-05-89
		US-A-	5037746	06-08-91
EP-A- 0387775	19-09-90	AU-A-	5130190	01-11-90
		CA-A-	2012312	16-09-90
		JP-A-	3130080	03-06-91

Letters patent

No. 649074

Patents Act 1990

STANDARD PATENT

I, Bruce Ian Murray, Commissioner of Patents, grant a Standard Patent with the following particulars:

Name and Address of Patentee:
Max-Planck-Gesellschaft Zur Forderung Der Wissenschaften E.V., Bunsenstrasse 10 D-3400 Gottingen Federal Republic Of Germany

Names of Actual Inventors: Fritz Eckstein; Olaf Heidenreich; Wolfgang A Pieken; Fritz Benseler; David B Olsen and David M. Williams

Title of Invention: Modified ribozymes

Application Number: 86139/91

Term of Letters Patent: Sixteen years commencing on 23 September 1991

Priority Details:

Number	Date	Country
EP9001731	12 October 1990	WORLD INTELLECTUAL PROPERTY ORGANIZATION (WIPO)

Dated this 30 day of August 1994

B.I.MURRAY
COMMISSIONER OF PATENTS

PATENTANWÄLTE

DIPL.-ING. H. WEICKMANN   DIPL.-PHYS. DR. K. FINCKE
DIPL.-ING. F. A. WEICKMANN   DIPL.-CHEM. B. HUBER
DR.-ING. H. LISKA   DIPL.-PHYS. DR. J. PRECHTEL
DIPL.-CHEM. DR. B. BÖHM   DIPL.-CHEM. DR. W. WEISS

POSTFACH 860 820
81635 MÜNCHEN
─────────
KOPERNIKUSSTRASSE 9
81679 MÜNCHEN

TELEFON (089) 4 55 63-0
TELEX 5 22 621
TELEFAX (089) 4 70 50 68

Max-Planck-Gesellschaft zur
Förderung der Wissenschaften
e.V.
Generalverwaltung
Postfach 101062
80084 München

cc: Max-Planck-Institut für biophysikalische Chemie
Herrn Prof. Eckstein, 37075 Göttingen
Ribozyme Pharmaceuticals Incorporated, Boulder
Lyon & Lyon, Los Angelos, Mr. Richard Warburg

IHR ZEICHEN / YOUR REF.	UNSER ZEICHEN / OUR REF.	DATUM / DATE
GI 1298 195/036	AUSL 12297/HBbj	September 27, 1994

Patentanmeldung Nr. 86139/91 in Australien RECEIVED

Modified Ribozymes

OCT - 4 1994   *Patent 1*

LYON & LYON
INTERNATIONAL DEPT.

Sehr geehrte Damen und Herren,

die Patentanmeldung Nr. 86139/91 in Australien ist zur Erteilung gekommen. Anliegend übersenden wir Ihnen die Patenturkunde mit der Bitte um Empfangsbestätigung.

Bitte beachten Sie die nachstehenden Daten:

Patent Nr.: **649074**

Beanspruchte Priorität: October 12, 1990   EP   PCT/EP90/01731

Beginn der Laufzeit: **September 23, 1991**

Längste Dauer: **16 ab dem 23.September 1991**

Fälligkeit der Jahresgebühren: jährlich am **23. September**

Wir werden Sie an die Fälligkeit der Jahresgebührenzahlungen rechtzeitig - jedoch ohne Haftung - erinnern.

Das Patent wurde am 30.August 1994 erteilt.

Mit freundlichen Grüßen

D. *Original Patent*
letter directly to
client.

Anlagen:
Patenturkunde

NUCLEOZYMES

Field of the Invention

This invention relates to nucleozymes, mixed nucleic acid polymers having catalytic activity. The invention also relates to methods of preparing and using nucleozymes.

Background of the Invention

Proteins were the only known catalysts of cellular reactions until the discovery of RNA catalysts (ribozymes). In some instances, the folded structure of a ribozyme catalyses a cleavage reaction on another part of the same molecule (cis-reaction). In other instances (trans-reaction), the ribozyme may also act as a catalyst on another RNA or DNA molecule (substrate) by cleaving or ligating pieces of the substrate without changing itself in the process. (Zaug et al. Science, Vol. 231, pp. 470-75, 1986; Cech Science, Vol. 236, pp. 1532-39, 1987).

A well-characterized example of a ribozyme is the self-splicing Group I intron from the nuclear rRNA of Tetrahymena thermophila. An intron is an intervening sequence in a eukaryotic gene which does not encode a protein or in rare cases encodes a different protein. Introns are transcribed along with coding sequences (exons) to produce precursor RNA. The introns are removed from the precursor RNA and the exons are ligated by RNA cleaving and splicing steps. The Group I intron or ribozyme of T. thermophila catalyzes its own removal from the precursor RNA molecule. (Kruger et al. Cell

- 2 -

31:147-157, (1982); Zaug et al. (1986)). The
self-splicing ribozyme catalyzes a variety of
phosphodiester transfer reactions. The ribozyme can
act as a ribonuclease, ligase, phosphotransferase,
acid phosphatase, polymerase and RNA restriction
endonuclease (Zaug, A.J., et al., Science 231:470-475
(1986); Zaug, A.J., et al., Nature 324:429-433
(1986); Zaug, A.J., et al., Biochemistry 25:4478-4482
(1986); Been, M.D., et al., Science 239:1412-1416
(1988); Doudna et al., Nature 339:519-522 (1989); all
incorporated by reference herein).

The "hammerhead" and "hairpin" ribozymes also
have been studied and described (Perreault et al.,
Nature 344:565-567 (1990); Perreault et al.
Biochemistry 30:4020-25 (1991); Yang et al.
Biochemistry 29:11156-60 (1990); Chowrira et al.
Biochemistry 30:8518-22 (1991); Uhlenbeck Nature,
328:596-600 (1987)). The hammerhead ribozyme forms a
stem loop secondary structure to form the
catalytically active molecule. The hairpin ribozyme
has a structure resembling a hairpin.

Although ribozymes are intriguing molecules,
their use for in vivo applications is limited if not
precluded. The all-RNA molecules are susceptible to
degradation from enzymes (RNAses) present in vivo.
There presently is no way known to inventors for
delivering such molecules to the intended site in an
active form.

- 3 -

Summary of the Invention

The present invention is based on the discovery that ribozymes have catalytically critical sites and that it is not necessary to have an all-RNA molecule to have catalytic activity. The ribozyme like molecules of the invention or "nucleozymes" have ribonucleotides or nucleic acid analogues (hereinafter NAAs) at catalytically critical sites and NAAs or deoxyribonucleotides at non-catalytically critical sites. The preferred nucleozymes have ribonucleotides at catalytically critical sites. Nucleozymes have catalytic activity on the same substrates as their ribozyme counterparts.

The nucleozymes of the present invention thus essentially are modified ribozymes having at least a portion, or all, of the ribonucleotides replaced with deoxyribonucleotides or NAAs. The nucleozymes are significantly more resistant to degradation than their all-RNA ribozyme counterparts because the chemicals or enzymes present in vivo do not recognize the nucleic acid internucleotide bonds. The resistance can be to either enzymatic or chemical degradation. Preferably, a majority of the ribonucleotides of the ribozyme are replaced with deoxyribonucleotides or NAAs. The stability of the nucleozymes allows them to be useful as therapeutic agents whereas ribozymes would be cleaved and rendered inactive by enzymes, e.g. RNAses, present in vivo.

- 4 -

The nucleozymes of the present invention are
chimeric nucleic acid polymers having catalytic
activity due to and preferably optimized by the
presence of RNA or a NAA at a catalytically critical
site. The present invention provides chemistry which
allows synthesis of the chimeric polymers and the
determination of catalytically critical sites. The
sites may be determined by varying the location of
deoxyribonucleotides in a chimeric polymer and
determining the locations responsible for or related
to the chimeric polymer's ability to catalyze.

The present invention also pertains to a method
for making a chimeric polymer. The polymers are made
by phosphitylating protected ribonucleotides or NAAs
units under conditions to form substantially
pure-protected phosphoramidites or synthons of a
single isomer. The protected phosphoramidites are
coupled to each other forming a protected chimeric
nucleic acid chain. The protecting groups are
removed from the chimeric nucleic acid chain under
conditions which completely deprotect the polymer.

The method of the present invention alleviates
problems associated with a known prior art method for
preparing chimeric RNA/DNA polymers (Perreault et al.
Nature 344:565-567 (1990); Wu et al. Journal of the
American Chemical Society 111:8531-33 (1989)). The
prior art synthetic method for making chimeric
polymers had problems with the migration of the
protecting groups during the phosphitylating step,
difficulty in removing the protecting groups and also

has problems resulting from the process of removing the protecting groups in the deprotection step. The former problem results in the production of monomer units having protecting groups in an undesired position. The latter problem results, in many cases, in a) nucleotide modification, b) phosphodiester linkage isomerization, and c) to retention of a substantial amount of protecting groups on the polymer resulting in a non-functional polymer. The first problem was overcome in the present invention by selecting a catalyst capable of minimizing migration of protecting groups, e.g., a combination of 2,4,6-collidine and N-methylimidazole. The problem in removing the protecting groups was eliminated by deprotecting the mixed polymer in the presence of ethanolic ammonia.

The present invention also pertains to methods of using the nucleozymes. The nucleozymes may be used to perform the same catalytic functions as their all-RNA ribozyme counterparts. For example, a nucleozyme may be used as a ribonuclease, ligase, phosphotransferase, acid phosphatase, polymerase, or an RNA restriction endonuclease. The nucleozymes may be used to selectively cleave and ligate substrates by contacting the substrates with a nucleozyme such that the nucleozyme targets a specific sequence in the substrate for cleavage or ligation. The nucleozymes may be used as polymerases to polymerize the production of an oligoribonucleotide or an oligodeoxyribonucleotide. The nucleozymes also may be used in place of antisense RNA technology.

- 6 -

The nucleozymes also may be used as therapeutic
agents introduced in vivo due to their resistance to
chemical and enzymatic degradation. The nucleozymes
may be used, for example, in a method for treating a
subject for a retrovirus associated disease, e.g.,
human immunodeficiency virus (HIV). The method
involves administering a therapeutically effective
amount of at least one nucleozyme to the subject such
that the nucleozyme cleaves the RNA genome of the
retrovirus rendering it inactive. A plurality of
nucleozymes also may be administered if it is
desirable to target more than one sequence in the RNA
genome.

A nucleozyme may be provided in a pharmaceutical
composition. The pharmaceutical composition would
include at least one nucleozyme and a
pharmaceutically acceptable carrier.

It is an object of the present invention to
provide a nucleozyme capable of maintaining its
catalytic properties in vivo.

It is an object of the present invention to
provide a chimeric nucleic acid polymer having
catalytic activity.

It is yet another object of the present
invention to provide a method for preparing chimeric
polymers which are free of protecting groups and
undesired isomeric side products.

It is yet another object of the present
invention to provide a homogenous chimeric polymer.

Brief Description of the Drawings

FIG 1 depicts a structure of a hammerhead nucleozyme.

FIG 2 is a table listing nucleozymes of the present invention indicating the positions of ribonucleotides in the nucleozymes. The position designations correspond to the structure depicted in FIG 1.

FIGs 3 and 4 are photographs of a gel demonstrating the cleavage of a $5'-^{32}P$-labeled RNA substrate. The designation "RNA" is the all-RNA containing ribozyme and the nucleozymes are as abbreviated in FIG 2.

FIG 5 is a photograph of a gel demonstrating the cleavage of a radioactive RNA substrate by nucleozymes of the present invention containing methoxy substituted NAAs. The nucleozymes are abbreviated as in FIG 2.

FIGs 6 and 7 are graphs depicting the stability of nucleozymes compared to the stability of the all-RNA ribozyme counterpart after exposure to both RNAse A (6) and a yeast cellular extract (7).

Detailed Description

The nucleozymes of the present invention have catalytic activity. Nucleozymes essentially are modified ribozymes preferably having at least one ribonucleotide or nucleic acid analogue (NAA) at a catalytically critical site(s) and deoxyribonucleotides or NAAs at non-critical sites.

The term "nucleozyme" is intended to include
catalytic chimeric polymeric chains containing
ribonucleotides and deoxyribonucleotides and/or
nucleic acid analogues.

A ribozyme is an all-RNA containing molecule
capable of being a biological catalyst. Ribozymes
are art recognized and examples of such molecules
include the "hammerhead" or "hairpin" ribozymes.

The term "catalytic activity" is intended to
include many types of catalysis. For example, the
catalytic activity could be that of a ribonuclease,
ligase, phosphotransferase acid, phosphatase,
polymerase, and RNA restriction endonuclease.

The term "nucleic acid analogue" (NAA) is
intended to include analogues which are structurally
similar to ribonucleotides or deoxyribonucleotides
and are capable of being monomer units in a polymer
capable of hybridizing with DNA or RNA. The analogue
may impart properties to a polymeric chain which
differ from those of a nucleotide but the analogue is
capable of being a monomer unit in a polymeric
chain. The NAA may impart resistance to chemical or
enzymatic degradation to the chimeric polymer. NAAs
may be selected for their structural conformation if
a particular conformation is desired for the
polymer. A NAA which is structurally similar to a
ribonucleotide may be positioned at a catalytically
site if the NAA is capable of participating and/or
attaining the desired catalytic activity.
Preferably, if the NAA is positioned at a

catalytically critical site it has a formula as
depicted in Formulas I-IV below wherein X is a good
coordinating ligand with divalent metal ions, eg.,
Mg^{+2}.

A nucleotide analogue may contain a heterocyclic
ring as depicted in the formulae I-IV below or may be
acyclic as shown in Formula V below. The preferred
heterocyclic ring containing analogues of the present
invention have three hydroxy substituents, alkoxy
substituents or combinations thereof.
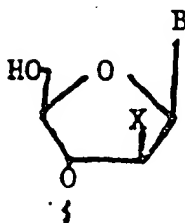
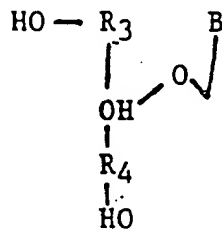Formula (I)   Formula (III)
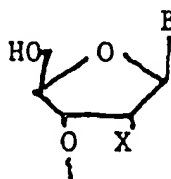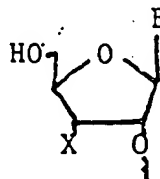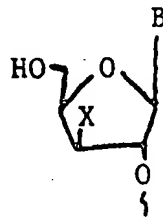
Formula (II)   Formula (V)   Formula (IV)

In the above formulae, B is a base. The base may be
substituted or unsubstituted. Examples of bases
include adenine, cytosine, guanine, uracil,

2-aminopurine, hypoxanthine, imidazole, $R_1-\overset{\overset{\textstyle O}{\|}}{C}-O-$,

- 10 -

R_1-SH-, and R_1-NH$_2$-. The term "base" is
art-recognized and one of ordinary skill in the art
would know which bases are useful in the present
invention. X is selected from the group consisting
of -OR$_1$, F, -R$_2$OH, -NH$_2$, -R$_2$NH$_2$, -Br,
-R$_2$Br and -R$_2$F wherein R$_1$ is a lower alkyl
group and R$_2$-R$_4$ are a lower alkyl hydrocarbon
chains. The term "lower alkyl" is intended to
include from one to six carbons, more preferably one
to three carbons and most preferably a single carbon
atom. For example, the most preferred X is methoxy.

B may be protected during the synthesis
process. The protecting groups may be the
conventional groups typically used in oligonucleotide
synthesis processes e.g., N^6-benzoyl for adenine,
N^4-benzoyl for cytosine, N^2-isobutyryl for
guanine, and N^2-benzoyl for 2-amiropurine. Other
useful protecting groups include phenoxyacetyl (PAC)
and t-butoxyacetyl (TAC). One of ordinary skill in
the art would know which protecting groups are
appropriate for a particular base.

The NAAs capable of being positioned at a
catalytically critical site may be determined by one
of ordinary skill in the art using the following
screening process. The catalytically critical sites
of a particular ribozyme may be determined by
positioning deoxyribonucleotides(s) at various
locations within the ribozyme and evaluating the
chimeric polymer's catalytic activity. After
determining the locations of the catalytically

critical sites, NAAs may be substituted for the
deoxyribonucleotide(s) and the chimeric polymer's
catalytic activity is again evaluated. If the NAA
containing chimeric polymer possesses catalytic
activity then the NAA is suitable for positioning at
a catalytically critical site.

The term "synthon" is intended to include the
fully protected monomer units (phosphoramidites) used
to assemble the nucleic acid analogues of a chimeric
polymer chain. The term "nucleic acid analogue" is
used to describe the units when polymerized as part
of a chimeric polymer chain.

The term "chimeric polymer" is intended to
include polymers containing at least two different
types of monomer units, e.g., RNA, DNA, or NAA. For
example, a chimeric polymer may include RNA/DNA,
RNA/NAA, or DNA/NAA polymeric chains. It should be
understood that the linkages between the building
units of the polymeric chain may be linkages capable
of bridging the units together for either _in vitro_ or
in vivo. For example, the linkage may be a
phosphorous containing linkage, e.g., phosphodiester
or phosphothioate, or may be a nitrogen containing
linkage, e.g., amide. It should further be
understood that the chimeric polymer may contain
non-nucleotide spacer molecules along with its other
nucleotide or analogue units. Examples of spacer
molecules which may be used are described in Nielsen
et al. _Science_, 254:1497-1500 (1991), the contents of
which are expressly incorporated by reference.

The analogues described above may be prepared
using synthetic methods capable of attaching the
desired moieties at the selected positions. Other
possible analogues will be apparent to one of
ordinary skill in the art. Deoxyribonucleotides are
readily available and may be purchased from a variety
of sources. The alkyl substituted heterocyclic ring
containing analogues may be synthesized by first
reacting the moiety being modified with arabino
triflates (trifluoromethane sulfonates) and second
displacing the arabino triflates with an appropriate
organotin compound selected on the basis of the
desired moiety. A schematic of this reaction is
depicted below:
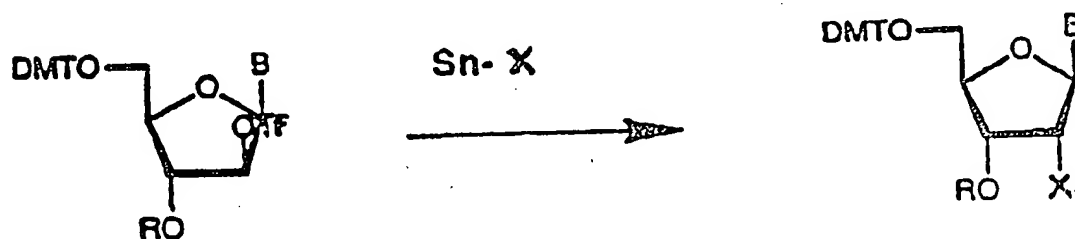
This synthesis scheme may be used to prepare
analogues wherein X is selected from the group
consisting of $-OR_1$, $-R_2OH$, R_2F, $-RBr$, and
$-RNH_2$. One of ordinary skill in the art would know
how to synthesis the halogenated analogues (X is $-F$
or $-Br$) and the amino substituted analogues (X is

- 13 -

-NH$_2$). These analogues may be synthesized as
described by Williams et al., Biochemistry,
30:4001-4009 (1991), Doerr et al., J. Org. Chem.,
32:1462 (1967), Mengel et al. Angew. Chem., 90:557
(1978), or Coddington et al., J. Org. Chem., 29:558
(1964), the contents of each of the references are
hereby expressly incorporated by reference.

The acyclic nucleic acid analogues may be
prepared by reacting a protected acyclic molecule
with a diol. Reaction processes which may be used
are described in Durand et al., Nucleic Acid
Research, 18:6353 (1990); Seela et al., Nucleic Acid
Research, 15:3113-3124 (1987); Cload et al., JACF,
113:6324-6326 (1991), the contents of each reference
is hereby expressly incorporated by reference.

The nucleozymes have catalytically critical
site(s) at which a ribonucleotide or NAA is necessary
for the nucleozyme to have the desired level of
catalytic activity. The term "catalytically critical
site" is intended to include sites which, if altered
from a ribonucleotide or a NAA to a
deoxyribonucleotide, substantially reduces or even
eliminates catalytic activity. A substantial
reduction in catalytic activity would be that
reduction which limits the usefulness of the
nucleozyme as a catalyst in vitro or in vivo. The
catalytically critical sites may be determined for
each nucleozyme. Catalytically critical sites can be
determined by preparing a variety of chimeric
polymers using the chemical techniques described

- 14 -

herein and comparing the catalytic activity of the
chimeric test polymers. The catalytically critical
sites in the all-RNA containing ribozyme counterparts
are determined by selecting sites believed to be
involved in catalysis and inserting a
deoxyribonucleotide at the particular site. If the
chimeric polymer does not have the same or
substantially the same catalytic activity as the
all-RNA counterpart, then the selected site is
presumed to be a catalytically critical site. The
hammerhead nucleozyme has four catalytically critical
sites which are the G9, G12, A13 and A29 positions
for the sequence depicted in FIG 1.

The nucleozymes of the present invention are of
a size capable of being synthesized using the
chemistry described herein. Preferably, the
nucleozymes have less than about 100 total building
units, more preferably, less than about 80 building
units, even more preferably, less than about 70
building units, and most preferably less than about
50 building units. Some nucleozymes may even have
less than about 20 building units. The preferred
nucleozyme is modeled after the hammerhead ribozyme,
the catalytic portion of which has 35 building
units. The term "building unit" is intended to
include ribonucleotides, deoxyribonucleotides, or
synthons.

The nucleozymes of the present invention are
modified ribozymes having at least a portion of the
ribonucleotides replaced with deoxyribonucleotides or

- 15 -

NAAs. The modified ribozymes or nucleozymes are
significantly more resistant to degradation than the
all-RNA counterparts. The degradation may be either
enzymatic or chemical degradation. The language
"significantly more resistant to degradation" is that
resistance which allows the nucleozyme to remain
largely intact for an extended period of time
relative to its all-RNA counterpart. Preferably it
has a resistance which allows it to be administered
for in vivo applications.

Resistance to enzymatic degradation may be
resistance to enzymes present in vivo, e.g. RNAses
such as RNAse A. FIGs. 6 and 4B are graphs depicting
the relationship between the percentage of the
nucleozyme being intact at a particular RNAse A (FIG.
6) or yeast cellular extract (FIG. 7) concentration.
The data set forth in FIGS. 6 and 7 was obtained
using the following procedure. A sample of 0.001
pmol of 5' labeled nucleozyme or ribozyme was
incubated with 0.5 mg of carrier TRNA in 50 mM
Tris-HCl (pH 7.4) and 10 mM Mg^{+2} and different
concentrations of RNAse A or yeast extract ten
minutes for A, thirty minutes for extract. The
reactions were stopped by the addition of 20 mM EDTA
and loaded and analyzed on 15% PAGE in 7M urea. The
yeast extract was prepared from a 1 ml culture of
yeast strain BWG2-9A grown to late-lag phase,
harvested and washed with 25 mM sodium phosphate
buffer (pH 7.8). The pellet was suspended in 100
μl of the same buffer and sonicated for 20 seconds

- 16 -

(60W). After centrifugation for five minutes in an
Eppendorf centrifuge, the supernatant was used
directly after appropriate dilutions and incubations
with the nucleozymes and the all-RNA ribozyme.

As shown in FIG. 6, the nucleozymes were at
least about 75% intact at an RNAse A log
concentration of -1.5 and at least about 80% intact
at an RNAse A log concentration of -2.5. Preferably,
the nucleozyme is at least about 90% intact at both
concentrations. The difference in stability between
the nucleozymes and ribozyme is even more apparent
when the enzyme is a yeast cellular extract (FIG.
7). The nucleozymes were at least 90% intact at all
of the tested concentrations.

The chemical degradation for purposes of this
invention is intended to include resistance to
chemicals present in vivo and in vitro. The
resistance may be to alkaline hydrolysis, e.g.,
sodium hydroxide and water.

The preferred nucleozymes of the present
invention are modified ribozymes having a majority of
the ribonucleotides replaced with
deoxyribonucleotides or NAAs. At least one of the
units is a deoxyribonucleotide and preferably most of
the units are deoxyribonucleotides. The nucleozymes
more preferably, have at least about 75%, even more
preferably at least about 85%, most preferably at
least about 90% of their ribonucleotides replaced
with deoxyribonucleotides or NAAs. The nucleozyme
also may be made up entirely of NAAs or a combination
of NAAs and deoxyribonucleotides.

The present invention also pertains to a method
of making a chimeric polymer. The method is similar
to that described by Scaringe et al. to produce an
all-RNA polymer. (<u>Nucleics Acid Research</u> Vol. 18,
No. 18, 5433-41 (1990)), the contents of which is
expressly incorporated by reference. The method
includes the steps of phosphitylating protected RNA,
DNA or NAA units under conditions which minimize
migration of the protecting group forming
isomerically pure protected phosphoramidites. The
protected RNA or synthons may have the desired
moieties protected with the protecting groups capable
of surviving the phosphitylation and coupling steps.
Examples of such groups include conventional DNA
protecting groups such as 5'-O-DMT, N-Bz (Ade and
Cyt), N-iBu (Gua), ß-cyanoethyl for phosphate, TB DMS
for 2'-hydroxyl.

The term "isomerically pure protected
phosphoramidites" is intended to include
phosphoramidite preparations free of a substantial
amount of undesired isomers of the phosphoramidites.
A substantial amount is that amount which would
substantially interfere or impede with the
preparation's ability to be used in forming a mixed
polymeric chain. For example, a consideration in the
chemical synthesis of a ribonucleotide
phosphoramidite is contamination of the desired
2'-O-protecting group-3'-O phosphoramidite with the
undesired 3'-O- protecting group
-2'-O-phosphoramidite. Syntheses performed with the

latter lead to oligonucleotides having 5'-2'
linkages. Isomerically pure protected
phosphoramidites of the present invention lead to
oligonucleotides which are free of such undesired
linkages.

The method of the present invention also
involves coupling the protected phosphoramidites
together forming a protected chimeric polymeric
chain. The coupling can be done using well-known
chemical techniques known to one of ordinary skill in
the art. Preferably the coupling is done on an
automated synthesizer.

After the chimeric polymeric chain is formed,
the protecting groups now can be removed from the
chain under conditions which completely deprotect the
polymer. Also, nucleotide base modification and/or
phosphodiester linkage isomerization of the chain now
can be minimized during the deprotection step.

The prevention of migration of the protecting
groups in the phosphitylation step can be
accomplished by phosphitylating in the presence of a
catalyst selected to minimize migration. An example
of such a catalyst is the combination of 2, 4,
6-collidine and N-methylimidazole.

The invention permits removal of the protecting
groups in a manner that is complete and that
minimizes nucleotide base modification and/or
phosphodiester linkage isomerization. Complete
removal of protecting groups includes substantially
complete removal where a polymeric chain may have a

small number of protecting groups still attached
which do not effect the polymeric chain's intended
catalytic function. The deprotection step is
accomplished by deprotecting the polymer in the
presence of an agent capable of minimizing such
effects. An example of such an agent is ethanolic
ammonia.

The method also allows the production of a
homogeneous RNA/DNA polymer free of undesired
isomeric products. The term "free" is intended to
include substantially free wherein a small amount of
protecting groups or undesired isomers are present as
long as the amount does not interfere or impede the
polymer's function. The minimization of nucleotide
base modification is intended to include that
modification which would effect a polymeric chain's
intended catalytic function. Minimizing
phosphodiester linkage isomerization, when used in
connection with a molecule, means preventing that
degree of isomerization which would, adversely affect
the molecule's intended catalytic function.
Minimizing phosphodiester linkage isomerization when
used in connection with a preparation, means
preventing that degree of isomerization which would
substantially affect the preparation's abilities to
be used for its intended catalytic functions.

The present invention also pertains to methods
for using the nucleozymes. The nucleozymes may be
used for any method in which a ribozyme presently may
be used. For example, the nucleozyme may be used to

selectively cleave an RNA substrate or to ligate two
pieces of RNA together. When cleaving a substrate,
the RNA substrate is contacted with at least one
nucleozyme which targets a specific sequence in the
substrate for cleavage. A plurality of nucleozymes
also may be used in a cleavage process.

The nucleozymes of the present invention also
may be used to polymerize an oligonucleotide
molecule. The oligonucleotide may contain
ribonucleotides, deoxyribonucleotides, and analogues
of deoxy- or ribonucleotides. A template is
contacted with a population of the appropriate
nucleotide monomer units and a nucleozyme under
conditions which allow an oligonucleotide
complementary to the template to form. The template
preferably is attached to a support.

The nucleozymes of the present invention also
may be used for therapeutic methods due to their
stability _in vivo_. The nucleozymes may be used, for
example, to treat a subject for a retrovirus
associated disease. A therapeutically effective
amount of at least one nucleozyme is administered to
a subject such that the nucleozyme(s) cleaves the RNA
genome of the retrovirus or the viral mRNA rendering
it inactive.

A retrovirus associated disease is intended to
include diseases involving retroviruses.
Retroviruses have an RNA genome making them
susceptible to cleavage by the at least one
nucleozyme or the plurality of nucleozymes. An
example of such a retrovirus associated disease is
AIDS wherein the causative retrovirus is the human
immunodeficiency virus (HIV).

- 21 -

The term "subject" is intended to include living organisms susceptible to retroviruses, e.g., mammals. Examples of subjects include humans, cats, and rats.

The language "therapeutically effective amount" is intended to include that amount capable of eliminating or significantly reducing the symptoms associated with retrovirus associated diseases. The amount may be determined on an individual basis and will be based, at least in part, on consideration of the severity of symptoms to be treated, the results sought and the size of the subject. Thus, a therapeutically effective amount may be determined by one of ordinary skill in the art employing such factors using no more than routine experimentation.

The nucleozymes of the present invention also may be used in conjunction with or in place of antisense RNA technology. That is, to control the expression of a gene by targeting an appropriate mRNA. A nucleozyme may be selected based on its ability to target a particular mRNA sequence and subsequently an effective amount of the nucleozyme may be administered to a subject. The effective amount would be that amount necessary to target the RNA and control expression of a selected gene.

The present invention also pertains to pharmaceutical compositions containing at least one nucleozyme and a pharmaceutically acceptable carrier. The language pharmaceutically acceptable carrier is intended to include carriers capable of being co-administered with the nucleozyme(s) while not adversely affecting the nucleozyme(s) catalytic

activity.   The carrier may be solid or liquid or a
gel.   Examples of liquid carriers include water, an
aqueous solution of a non-toxic salt, e.g., sterile
physiological saline solutions, or aqueous solutions
containing organic solvents, e.g., ethanol.   Also
suitable are emulsions, such as oil-in-water.   Solid
carriers may include nutritive carriers, e.g.,
sucrose or gelatin, or non-nutritive carriers, e.g.,
cellulose or talc.

The Preferred Embodiment

The preferred nucleozymes of the present
invention are modeled after the "hammerhead"
ribozyme.   The moderate size of the catalytic
hammerhead domains of RNA lends itself to chemical
synthesis.   As shown in FIG. 1, the conserved
hammerhead domain of around fifty ribonucleotides
found in naturally occurring RNA has been petitioned
between a thirty-five unit catalytic fragment 10, the
ribozyme, and a fourteen nucleotide substrate unit 12.

The all-DNA analog of the hammerhead domain is
inactive in catalysis.   It was determined that
nucleozymes containing as few as four ribonucleotides
out of a total of thirty five nucleotides have
catalytic activity.   Active hammerhead nucleozymes
require the presence of ribonucleotides in particular
at four positions, particular the G9, G12, A13, and
A29 positions shown in FIG. 2.

FIG. 2 is a table showing the chimeric polymers
prepared along with the positions of ribonucleotides
within the polymers.   Nucleozymes are abbreviated as
follows: DR and MR designate nucleozymes composed of

- 23 -

predominantly deoxyribonucleotides and 2'
-methoxynucleotides, respectively. The following
number indicates the number of ribonucleotides and
the final number refers to a particular combination
of ribonucleotides.

The all DNA analog of the hammerhead domain is
inactive in catalysis. Several RNA/DNA mixed
polymers appear as set forth and designated in the
table of FIG 2. As shown in FIGs 3 and 4, the mixed
polymer having ribonucleotides at positions 9, 10,
12, 13, 28, 29 and 30 of the ribozyme showed good
catalytic activity (FIG 3). The nucleozyme
containing the fewest ribonucleotides, DR4R3 and
DR4R2, showed at least some activity. In FIGs 3 and
4, S indicates the mobility of the intact substrate
and P, the product. RNA is the all-RNA ribozyme and
nucleozymes are abbreviated as in FIG 1. The
presence or absence of the enzymatic fragment is
indicated by + or -, respectively in FIG 3. Lane 1
is a control lacking the added catalytic fragment in
FIG 3. Lane 1 in FIG 4 is the 4.5 hour incubation of
the substrate with neither Mg^{+2} nor nucleozyme and
lane 2 is an incubation control in the presence of
Mg^{+2} but no added nucleozyme. The samples were
analyzed on 15% PAGE in 7M urea.

FIG 3 is a photograph showing the cleavage of
radioactive substrates by OMe-nucleozymes. The S
indicates the mobility of the intact substrate and P,
the product. The reactions were performed as in
FIG 2 except that the incubations of the substrate
were with the MR5R nucleozyme, for four hours and the
(Mg^{+2}) was varied. Lane 1 contained no Mg^{+2},

lane 2-10 mM, lane 3-20 mM, lane 4-30 mM and lane
5-50 mM.   The OMe-nucleozymes are nucleozymes having
synthons which are methoxysubstituted in the 2'
position.   The nucleozymes designated MR contain all
methoxysubstituted nucleozymes rather than
deoxyribonucleotides.

The catalytic activity of the nucleozymes was
further evaluated by determining parameters including
K_M and K_{cat} for some of the kinetic reactions.
In addition, the K_{Mg}, K_S and k_3 of nucleozyme
reactions were obtained to evaluate how extensive
deoxyribonucleotide substitution effects Mg^{+2}
cofactor binding.   A summary of these results is
presented in Table 1 below.

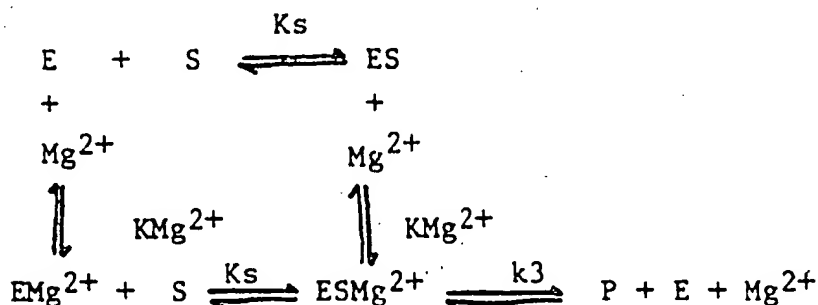TABLE I Kinetic parameters of nucleozyme-catalyzed
        reactions[a]

Substrate/ Nucleozyme	K_M (μM)	Kcat (1/min)	K_S (μM)	K_3 (1/min)	K_{Mg} (mM)
RS/DR4R3	4.2	0.004	5	0.013	23
RS/DR5R2	4.9	0.013	4	0.040	24
RS/DR7R	4.5	0.067	5	0.21	24
RS/RR	0.7	1.2	0.8	1.8	8.1

[a] The kinetic experiments were performed under the
standard conditions of FIG 2 with following
differences: the final concentrations were:
nucleozyme 0.05 μM; substrate from 0.5 μM to 5
μM; Mg^{2+} from 5 to 50 mM and the reaction times
from 30 min to 6 h at 30°C.   Initial rates were
determined from the first 10% of the reactions.   The
chronology of substrate and metal ion addition is

currently not known. The values for Ks, k3 and KMg
were, therefore, determined from the following random
assembly model for ternary complex formation:

The generalized reaction scheme used to
determine the K values is depicted below.

$$
\begin{array}{ccccc}
\text{E} & + & \text{S} & \xrightleftharpoons{\text{Ks}} & \text{ES} \\
+ & & & & + \\
\text{Mg}^{2+} & & & & \text{Mg}^{2+} \\
\Big\updownarrow\ \text{KMg}^{2+} & & & & \Big\updownarrow\ \text{KMg}^{2+} \\
\text{EMg}^{2+} + \text{S} & \xrightleftharpoons{\text{Ks}} & \text{ESMg}^{2+} & \xrightleftharpoons{\text{k3}} & \text{P} + \text{E} + \text{Mg}^{2+}
\end{array}
$$

The reaction scheme starts with the catalytic
species, the substrate and the Mg^{+2} cofactor,
traversing a ternary complex and yielding a product.
The apparent K_m's and K_s's of the three
nucleozymes are virtually identical and approximately
five times those of the all-RNA ribozyme acting on an
RNA substrate.

The chimeric polymers were synthesized as
follows:

Ribonucleotide, Deoxyribonucleotide, or 2'-Methoxy
Analogue Phosphoramidite Synthesis and Purification

Dry N-Acyl-5'-O-DMT-2'-O-sily-ribonucleoside (10
mmol, 1 eq) was dissolved in 30 ml dry THF in a 300
ml round bottom flask. 2, 4, 6-Collidine (75 mmol,
7.5 eq) was added followed by N-methylimidazole (5
mmol, 0.5 eq). (N,N-dispropylamino) (cyanoethyl)
phosphonamidic chloride (20 mmol, 2.0 eq [22 mmol,

2.2 eq in the case of guanosine or other nucleotide
bases with reactive lactams]) was then added dropwise
over 5 min at room temperature. The reaction was
clear at the start but a white precipitate formed
within 3 min (this is the collidine hydrochloride
salt). The reaction was complete after 1-2 hour
(determined by TLC). The reaction was then placed in
an ice bath and diluted with 100 ml (1 volume) ethyl
acetate. 150 ml 5% NaHCO$_3$ was added <u>slowly</u> (the
first 10 ml over 5 minutes). (In the case of
guanosine the reaction was first quenched with 5 ml
of absolute ethanol). Heat was generated by the
quenching of excess phosphitylating reagent. The
mixture was then transferred to a separatory funnel
and a second volume of ethyl acetate (100 ml) was
added after first rinsing out the reaction flask.
The aqueous phase was removed and the organic phase
washed with saturated NaCl. The combined aqueous
washes were back extracted with 50 ml. ethyl acetate
and the combined organic phases were dried over
Na$_2$SO$_4$. The solvent was removed <u>in vacuo</u>
yielding a viscous oil. Coevaporation (x 5) with 50
ml toluene afforded the crude phosphoramidite as an
offwhite foam or oil. Excess phosphinic acid and
collidine caused it to be oily. After leaving the
amidite under high vacuum overnight, resuspension and
rotovaping of the amidite with methylene chloride and
ethyl acetate usually produced a foam. The
phosphoramidites were further purified by silica gel
chromatography yielding a white foam in 75-85%

yields. This procedure was repeated for the
preparation of the other desired phosphoramidites
changing the initial protected nucleotide unit. The
phosphoramidites were used to synthesize the mixed
polymers using automated synthesis techniques
commonly used for the synthesis of
deoxyribonucleotides.

Automated Synthesis of Mixed Polymers

All syntheses were conducted on either a Gene
Assembler Plus (Pharmacia), or a Cyclone
(Milligen/Biosearch) synthesizer using standard
protocols with an extended 12 min coupling step. A
30 fold excess (150 µl of 0.1 M = 15 mg, ~ 15
µmol) of the phosphoramidites and a 400 fold excess
of tetrazole (400 µl of 0.5M = 200 µmol)
relative to CPG bound 5'-hydroxyl was used in each
coupling cycle. Synthesis scale was 0.5 µmol.
Average coupling yields on the Gene Assembler Plus,
monitored by an on-line colorimeter, were ~99.0%
and on the Cyclone 97-98%, determined by colorimetric
quantitation of the trityl fractions. Reaction
columns for 0.5 µmol syntheses were
Milligen/Biosearch 1.0 µmol columns.
Oligonucleotide synthesis reagents: 1) for GA plus:
detritylation solution was 2% TCA in ethylene
dichloride; capping was performed with 20% N-Methyl
imidazoyl in THF and 10% acetic anhydride/10%
2,6-lutidine in THF; oxidation solution was 0.02
MI_2, 1% lutidine, 10% water in tHF. Baker

- 28 -

Bio-Analyzed grade acetonitrile was further dried
over activated 4 A molecular sieves. Tetrazole
solution (0.5 M in acetonitrile was obtained from
Applied Biosystems. 2) for Cyclone: all standard DNA
synthesis ancillary reagents were used.

Deprotection of Mixed Polymers

The CPG-bound mixed polymer was transferred from
the synthesis column to a 4 ml glass screw top vial.
1 ml of ethanolic ammonia was added and heated at
55°C for 16 hr. After cooling to -20°C, the
ethanolic ammonia was removed from the CPG beads and
the CPG was washed with 0.5 ml of 50:50/ethanol:water
which was then added to the ethanolic ammonia. The
combined supernatants containing the
oligoribonucleotide were dried to a white powder. To
remove the silyl protecting groups, the
ammonia-deprotected mixed polymer was resuspended in
50 µl of 50:50/ethanol:water and 600 µl of 1M
TBAF/THF and left at room temperature for about 24
hr. The solution was then added directly to 10 ml of
0.1M TEAB and loaded onto a Qiagen 500 anion exchange
cartridge (Qiagen Inc., Studio City, CA) prewashed
with 10 µl of 0.05M TEAB, the nucleozyme was eluted
with 7 ml of 2M TEAB and dried down to a white powder.

Gel Purification of Fully Deprotected Mixed Polymers

The oligomers were first checked by analytical
PAGE (0.75 mm x 20 cm x 45 cm). 1 ODU of
oligonucleotide in 5 µl H_2O was added to 5 µl

of deionized formamide and the total 10 µl solution
was loaded into a 1 cm wide lane. Following
electrophoresis the gels were photographed by placing
the gel over a fluorescent TLC plate and illuminating
the gel with a UV lamp. The desired sequence was
established according to electrophoretic mobility and
purified by preparative electrophoresis using 1.5 mm
thick gels with a single 12 cm wide lane. After
electrophoresis the desired band was excised,
crushed, placed into a sterile 5 ml test tube, and
covered with 50 mM NH$_4$OAc pH 7.0. The tube was
covered and kept at 37°C O/N. The supernatant was
then removed and the gel pieces washed with an
additional 1 ml of the extraction buffer. The
combined washings were filtered through a 0.45 micron
filter and loaded onto a 1 gram size Sep-Pak C18
cartridge (Waters-Millipore) prewashed with 5 ml each
of acetonitrile, 50% acetonitrile/0.1M TEAB and 0.1M
TEAB. After washing the cartridge with 5 ml of 0.1 M
TEAB, the RNA was eluted in 5 ml 35:35:30
acetonitrile/methanol/water and dried down to a white
powder.

The mixed polymers set forth in the Table of
FIG 2 were prepared as described above.

EQUIVALENTS

Those skilled in the art will be able to
ascertain, using no more than routine
experimentation, many equivalents of the specific
embodiments of the invention described herein.

- 30 -

These and all other equivalents are intended to be encompassed by the following claims.

What is claimed is:

CLAIMS

1. A nucleozyme having catalytic activity,
comprising:

a ribonucleotide and at least one second
unit being a deoxyribonucleotide or nucleic acid
analogue, the nucleozyme having a ribonucleotide or
nucleic acid analogue at a catalytically critical
site.

2. The nucleozyme as claimed in claim 1
including at least one deoxyribonucleotide.

3. The nucleozyme as claimed in claim 2
wherein all of the second units are
deoxyribonucleotides.

4. A nucleozyme as claimed in claim 1 wherein
the second units include at least one nucleic acid
analogue.

5. A nucleozyme as claimed in claim 1 wherein
the second units include at least 50% nucleic acid
analogues.

6. A nucleozyme as claimed in claim 4 wherein
the analogue contains a heterocylic ring.

7. A nucleozyme as claimed in claim 4 wherein
the analogue is acyclic.

- 32 -

8.   A nucleozyme as claimed in claim 4 wherein the analogue has the following formula:

$$HO - R_3 \quad \overset{B}{\underset{\displaystyle \underset{OH}{|}}{}}$$
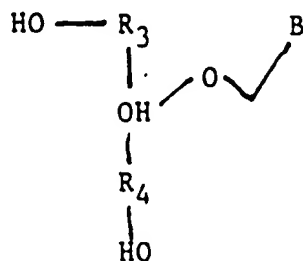
wherein B is a base and R_3 and R_4 are each independently lower alkyl groups.

9.   A nucleozyme as claimed in claim 8 wherein B is selected from the group of adenine, cytosine, guanine, uracil, 2-aminopurine,
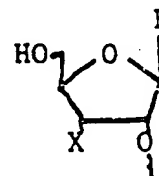
hypoxanthine, and imidazoyl, $R_1\overset{\displaystyle O}{\overset{\|}{C}}-O-$, R_1-SH-, R_1-NH_2- wherein R_1 is a lower alkyl group.

I0.   A nucleozyme as claimed in claim 4 wherein the ribonucleotide analogue has a formula selected from the group consisting of:
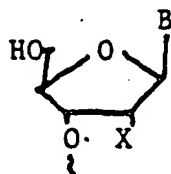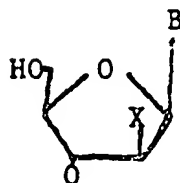
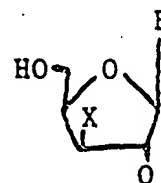Formula (I)   Formula (III)

Formula (II)   Formula (IV)

- 33 -

wherein B is a base and X is a good
coordinating ligand for a divalent metal ion.

11.   A nucleozyme as claimed in claim 10 wherein
B is selected from the group consisting of adenine,
cytosine, guanine, uracil, 2-aminopurine,
hypoxanthine, and imidazoyl;
and X is selected from the group consisting of
$-OR_1$, F, $-R_2OH$, and $-CH_2F$, wherein R_1 is a
lower alkyl group and R_2 is a lower alkyl chain.

12.   A nucleozyme as claimed in claim 10 wherein
the analogue has a formula as shown in (I) and X is
methoxy.

13.   A nucleozyme as claimed in claim 1 wherein
the nucleozyme has a ribonucleotide at a plurality of
catalytically critical sites.

14.   A nucleozyme as claimed in claim 1 wherein
the nucleozyme has less than about 100 total building
units.

15.   A nucleozyme as claimed in claim 14 wherein
the nucleozyme has less than about 80 total building
units.

16.   A nucleozyme as claimed in claim 15 wherein
the nucleozyme has less than about 70 total building
units.

- 34 -

17.   A nucleozyme as claimed in claim 16 wherein
the nucleozyme has less than about 50 total building
units.

18.   A nucleozyme as claimed in claim 1 wherein
the nucleozyme has a sequence corresponding to the
ribozyme selected from the group consisting of
hammerhead and hairpin.

19.   A nucleozyme as claimed in claim 18 wherein
the ribozyme is the hammerhead ribozyme.

20.   A nucleozyme as claimed in claim 19 wherein
the catalytically critical sites are the G9, G12,
A13, and A29 positions.

21.   A nucleozyme as claimed in claim 1 wherein
the nucleozyme is selected from the group consisting
of DR4R2, DR4R3, DR5R2, DR5R3, DR6R2, DR7R, MR5R2,
MR3R, and MR2R.

22.   A nucleozyme having catalytic activity,
comprising:
        a modified ribozyme having at least a
portion of its ribonucleotides replaced with
deoxyribonucleotides or nucleic acid analogs, the
modified ribozyme being significantly more resistant
to degradation than its all-RNA counterpart.

23. A nucleozyme as claimed in claim 22 wherein the resistance is to enzymatic degradation.

24. A nucleozyme as claimed in claim 22 wherein the resistance is to chemical degradation.

25. A nucleozyme as claimed in claim 22 or 23 wherein the modified ribozyme is a modified hammerhead ribozyme.

26. A nucleozyme as claimed in claim 22 wherein the resistance is to enzymatic degradation and the nucleozyme is at least 80% intact at an RNase A log concentration of -2.5.

27. A nucleozyme as claimed in claim 26 wherein the nucleozyme is at least 90% intact.

28. A nucleozyme as claimed in claim 22 wherein the resistance is to enzymatic degradation and the nucleozyme is at least 75% intact at an RNase A log concentration of -1.5.

29. A nucleozyme as claimed in claim 28 wherein the nucleozyme is at least 90% intact.

30. A nucleozyme as claimed in claim 25 wherein the resistance is to enzymatic degradation and the nucleozyme is at least 80% intact at an RNase A log concentration of -2.5.

31.   A nucleozyme as claimed in claim 30 wherein the nucleozyme is at least 90% intact.

32.   A nucleozyme as claimed in claim 25 wherein the resistance is to enzymatic degradation and the nucleozyme is at least 75% intact and an RNase A log concentration of -1.5.

33.   A nucleozyme as claimed in claim 32 wherein the nucleozyme is at least 90% intact.

34.   A nucleozyme having catalytic activity comprising

a chimeric polymer made up of RNA and second units being deoxyribonucleotides or nucleic acid analogues, said chimeric polymer having catalytic activity and having a majority of its total units being deoxyribonucleotides or nucleic acid analogs.

35.   A nucleozyme as claimed in claim 34 wherein at least one of the second units is a deoxyribonucleotide.

36.   A nucleozyme as claimed in claim 34 wherein all of the second units are deoxyribonucleotides.

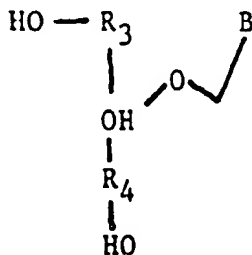37.   A nucleozyme as claimed in claim 34 wherein the second units include a nucleic acid analogue.

- 37 -

38.   A nucleozyme as claimed in claim 37 wherein the second units include a plurality of nucleic acid analogues.

39.   A nucleozyme as claimed in claim 37 wherein at least one of the nucleic acid analogues contains a heterocyclic ring.

40.   A nucleozyme as claimed in claim 37 wherein the nucleic acid analogue is acyclic.

41.   A nucleozyme as claimed in claim 38 wherein the analogue has the following formula:

$$HO - R_3 \quad\quad B$$

$$\begin{array}{c} | \\ OH \\ | \\ R_4 \\ | \\ HO \end{array}$$

wherein B is a base and R_3 and R_4 are each independently lower alkyl groups.

42.   A nucleozyme as claimed in claim 39 wherein the base is selected from the group of adenine, cytosine, guanine, uracil, 2-aminopurine,
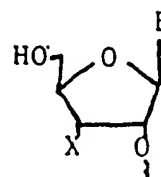
$$O$$
$$\|$$
hypoxanthine, and imidazoyl R_1C-O-, R_1-SH-, R_1-NH_2- wherein R_1 is a lower alkyl group.

43.   A nucleozyme as claimed in claim 37 wherein
the analogue has a formula selected from the group
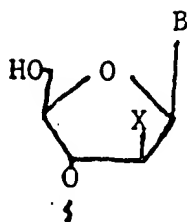consisting of:
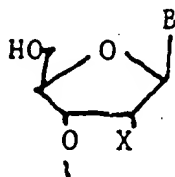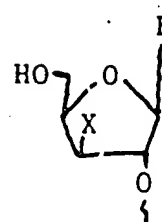
Formula (I)

Formula (III)

Formula (II)

Formula (IV)

wherein B is a base and X is a good
coordinating ligand with a divalent metal ion.

44.   A nucleozyme as claimed in claim 40 wherein
the base is selected from the group consisting of
adenine, cytosine, guanine, uracil, 2-aminopurine,
hypoxanthine, and imidazoyl;
    and X is selected from the group consisting of
$-OR_1$, F, $-R_2OH$, and $-CH_2F$, wherein R_1 is a
lower alkyl group and R_2 is a lower alkyl chain.

45.   A nucleozyme as claimed in claim 43 wherein
the analogue has a formula as shown in (I) and X is
methoxy.

46.   A nucleozyme as claimed in claim 34 wherein
all of the second units are 2'-methoxy ribonucleotide
analogues.

- 39 -

47.   A nucleozyme as claimed in claim 34 wherein the nucleozyme is a modified hammerhead ribozyme.

48.   A nucleozyme as claimed in claim 34 or 47 wherein at least 50% of the polymer is second units.

49.   A nucleozyme as claimed in claim 48 wherein at least 75% of the polymer is second units.

50.   A nucleozyme as claimed in claim 49 wherein at least 85% of the polymer is second units.

51.   A nucleozyme as claimed in claim 50 wherein at least 90% of the polymer is second units.

52.   A chimeric polymer including at least one ribonucleotide and at least one second unit being a deoxyribonucleotide or nucleic acid analogue, the polymer having catalytic activity optimized by the presence of RNA at a catalytically critical site.

53.   A chimeric polymer as claimed in claim 52 wherein the second units include a deoxyribonucleotide.

54.   A chimeric polymer as claimed in claim 52 wherein RNA is present at all of the catalytically critical sites.

- 40 -

55.   A chimeric polymer as claimed in claim 53
wherein all of the second units are
deoxyribonucleotides.

56.   A method of making a chimeric polymer
comprising:

      phosphitylating protected RNA or second
units under conditions to form substantially
pure-protected phosphoramidites of a single isomer,
the second units being deoxyribonucleotides or
nucleic acid analogues;

      coupling the protected phosphoramidites
together forming a protected chimeric polymer chain;
and

      deprotecting the chimeric polymer chain
under conditions which completely deprotect the
chimeric polymer.

57.   A method as claimed in claim 56 wherein the
second units in the phosphitylating step include a
deoxyribonucleotide.

58.   The method as claimed in claim 56 or 57
wherein the phosphitylating agent in the
phosphitylating step is (N,M-disopropyl amino)
(cyanoethyl) phosphonamidic chloride.

59.   A method as claimed in claim 56 or 57
wherein the phosphitylating step is conducted in the
presence of 2,4,6-collidine and N-methylimidazole.

60.   A method as claimed in claim 58 wherein the
phosphitylating step is conducted in the presence of
2,4,6-collidine and N-methylimidazole.

61.   A method as claimed in claim 56 wherein the
deprotecting step is conducted in the presence of
ethanolic ammonia.

62.   A method as claimed in claim 59 wherein the
deprotecting step is conducted in the presence of
ethanolic ammonia.

63.   A chimeric polymer containing
ribonucleotide units and second units joined together
forming a chimeric polymer, the second units being at
least one deoxyribonucleotide or nucleic acid
analogue and the chimeric polymer being free of
protecting groups.

64.   A chimeric polymer as claimed in claim 63
wherein the second units include deoxyribonucleotides.

65.   A homogeneous RNA/DNA polymer containing
both deoxyribonucleotide and ribonucleotide units
joined together forming a mixed polymer, the mixed
polymer being free of undesired isomeric products.

66.   A homogeneous polymer as claimed in claim
65 wherein the undesired isomeric products contain
5-2 linkages.

67.   In a chimeric nucleotide polymer synthesis,
the improvement comprising, the use of a catalyst
capable of minimizing migration of protecting groups
during a phosphitylation step.

68.   A method as claimed in claim 67 wherein the
catalyst is a combination of 2,4,6-collidine and
N-methylimidazole.

69.   In a chimeric nucleotide polymer synthesis,
the improvement comprising, the removal of protecting
groups from a protected mixed polymer in the presence
of ethanolic ammonia.

70.   A method for selectively cleaving an RNA
substrate, comprising:
        contacting a substrate with a nucleozyme
which targets a specific sequence in the substrate
for cleavage.

71.   A method for treating a subject for a
retrovirus associated disease, comprising:
        administering a therapeutically effective
amount of a nucleozyme to the subject such that the
nucleozyme cleaves the RNA genome of the retrovirus
or viral m RNA rendering it inactive.

72.   A method as claimed in claim 71 wherein a
plurality of nucleozymes are administered.

- 43 -

73.   A method as claimed in claim 71 wherein the retrovirus is human immunodeficiency virus.

74.   A pharmaceutical composition, comprising:
       at least one nucleozyme; and
       a pharmaceutically acceptable carrier.

75.   A method of polymerizing an oligonucleotide molecule comprising:
       contacting a template with a population of nucleotide monomer units and a nucleozyme such that an oligonucleotide complementary to the template is formed.

76.   A method as claimed in claim 75 wherein the oligonucleotide formed is an oligoribonucleotide.

77.   A method as claimed in claim 75 wherein the oligonucleotide formed is an oligodeoxyribonucleotide.

78.   A method as claimed in claim 70 wherein the RNA is messenger RNA.

79.   A method for controlling the expression of a gene in a subject comprising:
       selecting a nucleozyme which targets an RNA sequence involved in the expression of a gene;
       administering an effective amount of the selected nucleozyme to the subject such that the nucleozyme targets the RNA sequence and inhibits the expression of a gene.

FIG 1

Nucleozyme
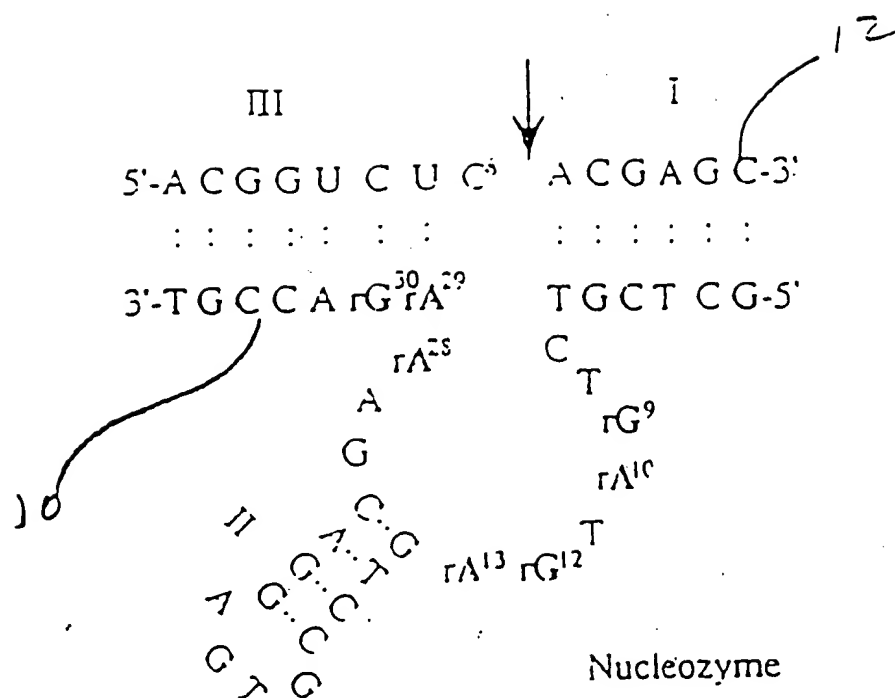

FIG 2

	Position of ribonucleotide		
	$rGrA^{9,10}$	$rGrA^{12,13}$	$rArArG^{28,29,30}$
Nucleozyme			
DR2R	rG	rA	– –
DR3R	rG	rA	rA
DR4R1	rG	rA	rArG
DR4R2	rG	rGrA	rG
DR4R3	rG	rGrA	rA
DR5R2	rG	rGrA	rArG
DR5R3	rGrA	rGrA	rG
DR6R2	rGrA	rGrA	rArG
DR7R	rGrA	rGrA	rArArG
OMe-nucleozyme			
MR2R	rG	rA	– –
MR3R	rG	rA	rA
MR5R	rG	rGrA	rArG

FIG 3
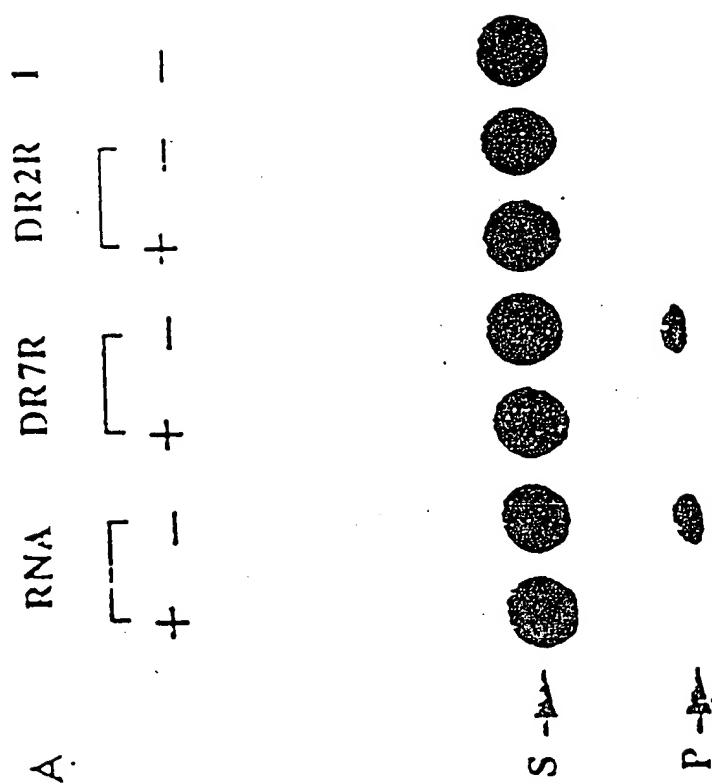
FIG 4
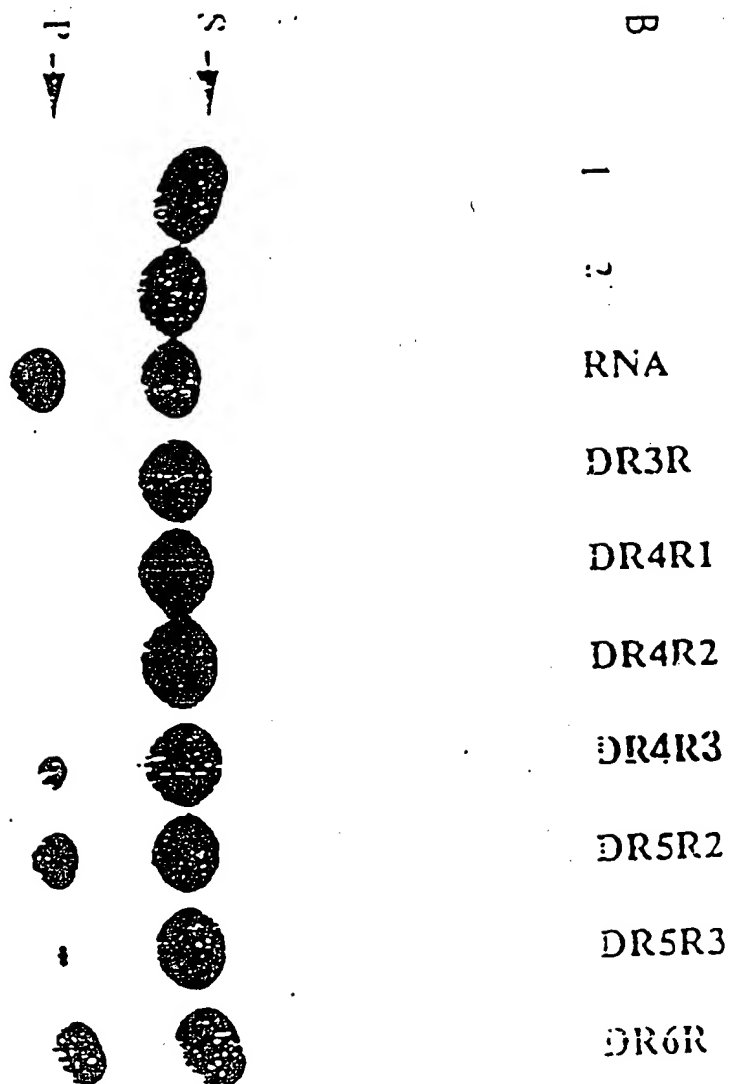
FIG 5
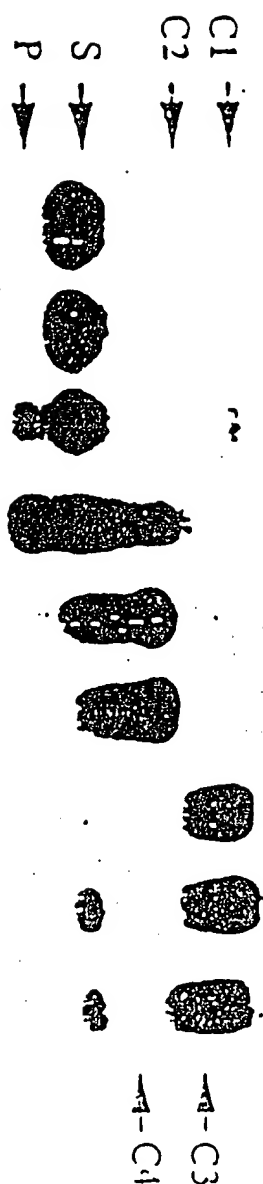
FIG 6

6 / 6

FIG 7

INTERNATIONAL SEARCH REPORT

International Application No   PCT/US   93/00833

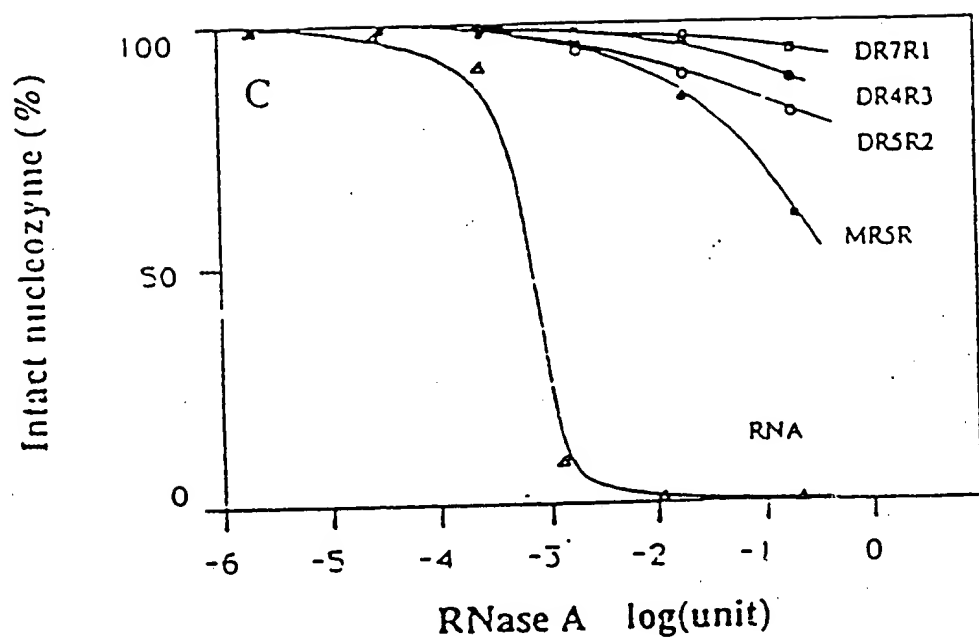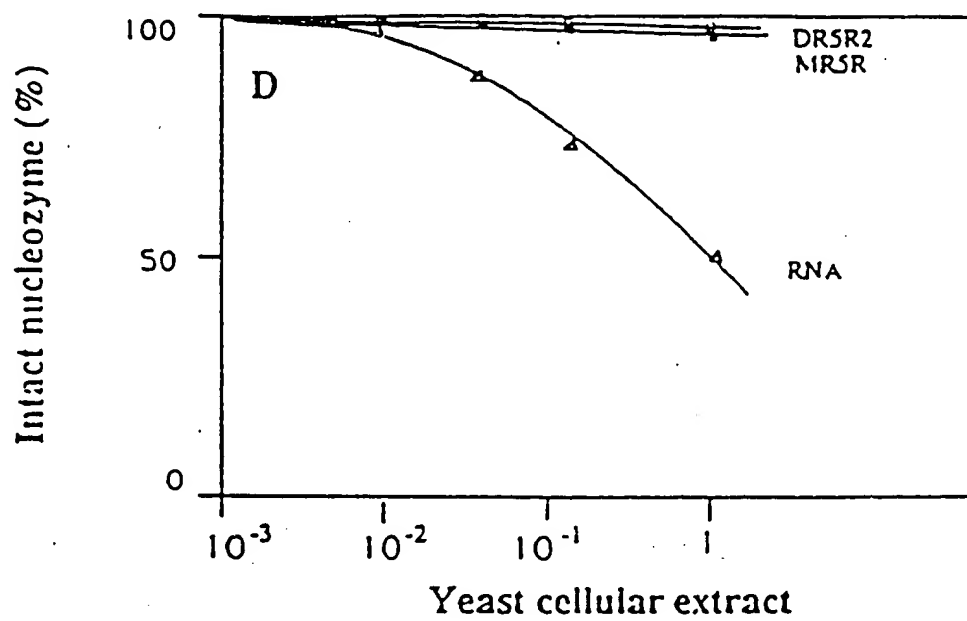## I. CLASSIFICATION OF SUBJECT MATTER   (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N9/00;   A61K31/70;   C07H21/00;   C12P19/34

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K ; C07H

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 103 162 (CITY OF HOPE) 21 March 1991 see the whole document	1-3, 13-20 22-23, 25-36, 47-57, 63-66, 70-71 73-74, 78-79
Y		56-62, 67-69

	-/--	

eXX

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 MAY 1993	03. 06. 93

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	NUCLEIC ACIDS RESEARCH vol. 18, no. 18, 25 September 1990, ARLINGTON, VIRGINIA US pages 5433 - 5441 SCARINGE, S.A. ET AL. 'Chemical synthesis of biologically active oligoribonucleotides using beta-cyanoethyl prtected ribonucleoside phosphoramidites' cited in the application	56-62
Y	see the whole document ---	56-62, 67-69
X	SCIENCE vol. 253, 19 July 1991, LANCASTER, PA US pages 314 - 317 PIEKEN, W.A. ET AL. 'Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes' see the whole document ---	1,4-6, 10-11, 13-20, 22-23, 25-34, 37-39 42-43, 47-48, 52,54, 56,63,70
X	BIOCHEMISTRY vol. 30, no. 16, 1991, EASTON, PA US pages 4020 - 4025 PERREAULT, J.-P. ET AL. 'Relationship between 2'-hydroxyls and magnesium binding in the hammerhead RNA domain: a model for ribozyme catalysis' cited in the application see the whole document ---	1-3, 13-20, 34-35, 47, 52-54, 63-66,70
X	WO,A,8 804 300 (UNIVERSITY PATENTS, INC.) 16 June 1988 see page 16, line 10 - page 17, line 24; claim 72 ---	75-76
X	SCIENCE vol. 251, 20 March 1991, LANCASTER, PA US pages 1605 - 1608 DOUDNA, J.A. ET AL. 'A multisubunit ribozyme that is a catalyst of and template for complementary strand RNA synthesis' see the whole document ---	75-76

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)	
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.

Category	Citation	Relevant to Claim No.
P,X	BIOCHEMISTRY vol. 31, no. 21, 2 June 1992, EASTON, PA US pages 5005 - 5009 YANG, J.-H. ET AL. 'Minimum ribonucleotide requirement for catalysis by the RNA hammerhead domain' see the whole document ---	1,4-6, 10-34, 37-39, 42-52, 54,56, 58-63 67-70
P,X	EMBO JOURNAL vol. 11, no. 5, May 1992, EYNSHAM, OXFORD GB pages 1913 - 1919 PAOLELLA, G. ET AL. 'Nuclease resistant ribozymes with high catalytic activity' see the whole document ---	1,4-6, 10-20, 22-34, 37-39, 42-43, 45-52,63 70
P,X	WO,A,9 207 065 (MAX-PLANCK GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 30 April 1992 see page 4, line 6 - page 7, line 3 see claims; example 4	1,4-6, 10-1! 13-20 22-34, 37-39, 42-44 47-52, 54,63, 70-71, 73-79